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FIGURE 1

Polymerase Chain Reaction (PCR)

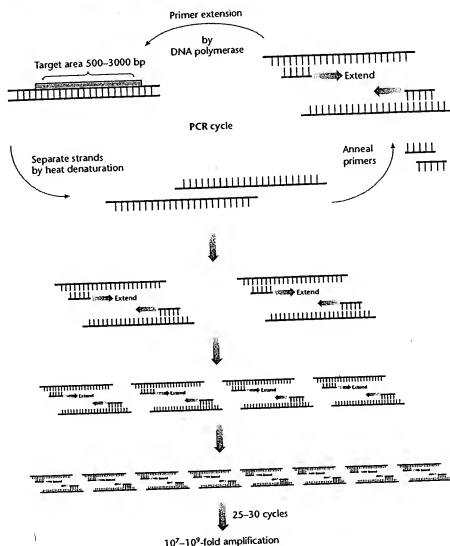


Figure 1 The PCR amplification cycle.

primer sequences, but manual inspection of the oligonucleotide is still necessary to maximize successful PCR amplifications.

The concentrations of the PCR cocktail ingredients are also important for product specificity, fidelity and yield. In addition to *Taq* DNA polymerase and primers, the PCR mixture contains the cofactor magnesium ion (Mg^{2+}), the four 2'-deoxyribonucleoside-5'-triphosphates (dNTPs) and the buffer. In general, PCR reagent concentrations that are too high from 'standard conditions' result in nonspecific products with high misincorporation errors, and those that are too low result in insufficient product. A typical 50- μ l PCR cocktail that contains 0.4 μ mol L^{-1} of each primer, 200 μ mol L^{-1} of each dNTP, 1.5 mmol L^{-1} $MgCl_2$, and 1.25 units *Taq* DNA polymerase in

10 mmol L^{-1} tris-HCl, pH 8.3, 50 mmol L^{-1} KCl buffer works well for most PCR applications. The optimal Mg^{2+} concentration, however, may need to be determined empirically for difficult target templates. The performance and fidelity of *Taq* DNA polymerase are sensitive to the free Mg^{2+} concentration (Eckert and Kunkel, 1990), which ionically interacts with not only the dNTPs but also with the primers, the template DNA, ethylenediaminetetraacetic acid (EDTA), and other chelating agents. In most cases, the Mg^{2+} concentration will range from 1.0 to 4.0 mmol L^{-1} .

The number of cycles and the cycle temperature/length of time for template denaturation and primer annealing and extension are important parameters for high-quality PCR results. The optimal number of cycles is dependent on

MOLECULAR AND FUNCTIONAL CHARACTERIZATION OF ANGIOTENSIN II AT2 RECEPTOR IN NEUROBLASTOMA N1E-115 CELLS

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Daniel Lazard, Phi Villageois, Nathalie Elbaz, and A. Donny Strosberg

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France

INTRODUCTION

Since the discovery in 1989 of a second subtype (AT₂) of angiotensin II (Ang II) receptors (1, 2), its functional significance has remained a matter of debate. Expression of AT₂ receptors was detected in adrenals, uterus, ovary, heart, brain, and at highest levels in the developing fetus. This subtype has been associated with reduction of intracellular cGMP levels (3-5), modulation of tyrosine phosphorylation (4, 6-9), inhibition of a T-type calcium channel (10, 11), opening of potassium channels (12, 13), release of prostaglandin (14) and production of arachidonic acid (15). Recent molecular cloning of the AT₂ receptor cDNA in rat (6, 16), mouse (17) and human (18, 19) revealed a structural organization typical of G-protein coupled receptors, but did not provide yet a clear understanding of transduction pathways and physiological functions associated with this subtype.

To address the question of AT₂ signaling, we have undertaken a detailed pharmacological, molecular and functional analysis of this receptor subtype in the murine neuroblastoma cell line N1E-115 (9). These cells express a single molecular class of AT₂ receptors and no detectable AT₁ binding site. In these cells, AT₂ receptors are functionally coupled to protein tyrosine dephosphorylation. The N1E-115 cell line thus constitutes a simple and well-characterized cellular model for further analyses of AT₂ receptor regulation and function.

PHARMACOLOGICAL CHARACTERIZATION OF ANG II RECEPTORS IN N115 CELLS

Subtypes (AT₁ and AT₂) of Ang II receptors expressed in neuroblastoma N1E-115 cells were analyzed using the non-selective iodinated Ang II antagonist: ¹²⁵I-(Sar, Ile)-AngII,

Recent Advances in Cellular and Molecular Aspects of Angiotensin Receptors
Edited by Mohan K. Raizada et al., Plenum Press, New York, 1996

167

Table I. Binding parameters of various ligands to membranes of N1E-115 cells and COS-AT2 cells. Values are from Nahmias et al. (9). n.d. stands for non determined

Radioligand	N1E-115		COS-AT2	
	Kd(pM)	Bmax(fmol/mg)	Kd(pM)	Bmax(fmol/mg)
¹²⁵ I-(Sar, Ile)AngII	233±33	298±53	n.d.	n.d.
¹²⁵ I-CGP 42112	91±19	321±42	75±4	125±11
Competitor	IC50 (nM)		IC50 (nM)	
(Sar, Ile)AngII	0.45±0.09		0.39±0.07	
CGP 42112	0.53±0.15		0.45±0.08	
Ang II	1.22±0.12		0.84±0.28	
PD 123319	4.60±0.21		4.74±0.55	
DUP 753	> 10,000		> 10,000	

and the AT2-selective radioligand ¹²⁵I-CGP 42112. Scatchard analyses revealed that both ligands bound to a single class of high affinity binding sites with the same Bmax (Table I), indicating that most if not all Ang II receptors were of the AT2 subtype. The absence of the AT1 subtype was confirmed by the inefficiency of the AT1-selective ligand DUP 753 to compete for binding of ¹²⁵I-(Sar, Ile)-AngII (Table I). In contrast, the AT2-selective ligands CGP 42112 and PD 123319 were able to totally displace the binding of ¹²⁵I-(Sar, Ile)-AngII in monophasic curves, indicating the presence of a single pharmacological class of AT2 receptors, which binding parameters are presented in Table I.

Fluharty and colleagues (20, 21) previously reported expression of both AT1 and AT2 receptor subtypes in N1E-115 cells. More recently, these authors identified two subpopulations of AT2 receptors (designated as peak I and peak III, respectively) that could be separated by heparine sepharose chromatography (22). These two populations differed in their affinity for the AT2-selective ligand PD 123319, their sensitivity to DTT and to analogs of GTP, and their reactivity towards a polyclonal anti-AT2 receptor antiserum. Discrepancy between these data and ours may be explained by the divergence occurring in cell lines that were independently passaged for many years under different culture conditions. The AT2 subtype expressed in our cell line shows moderate affinity for PD 123319, increased binding in the presence of reducing reagent and insensitivity to GTPγS (9), and would thus correspond to the so-called "peak III".

Cloning of AT2 receptor cDNA from N1E-115

For a further molecular characterization, cDNA clones were isolated from a N1E-115 cDNA library by homology screening, using a DNA probe derived by a PCR strategy on the basis of the rat AT2 receptor sequence (9). Nucleotide sequencing revealed that AT2 cDNA clones from N1E-115 belong to the same molecular subtype as those isolated from rat (16) and mouse (17) fetal tissues, as well as from rat pheochromocytoma PC12W cells (6), human myometrium (18) and human lung (19).

One remarkable feature of the mouse AT2 cDNA sequence is the presence of two in-frame initiator ATG codons that potentially generate two polypeptides of different length (Fig. 1). The downstream ATG codon is more likely utilized, as its position is in agreement with Kozak's consensus for initiation of translation (23), and corresponds to the single initiator ATG codon present in the rat (6, 16) and the human (18, 19) AT2 sequences. It cannot be excluded however that in certain conditions, translation is initiated at the upstream ATG

[illegible]

Figure 1. Nucleotide and deduced amino-acid sequences of the AT2 cDNA from NIE-115 cells. Two potential initiator ATG codons are boxed. Amino-acid number one corresponds to the downstream initiator ATG codon (position 157 of the nucleotide sequence). The seven transmembrane segments (tm1-tm7) are highlighted by solid bars. Potential N-linked glycosylation sites are indicated by stars. Putative sites for phosphorylation by protein kinase A and protein kinase C are indicated by triangles.

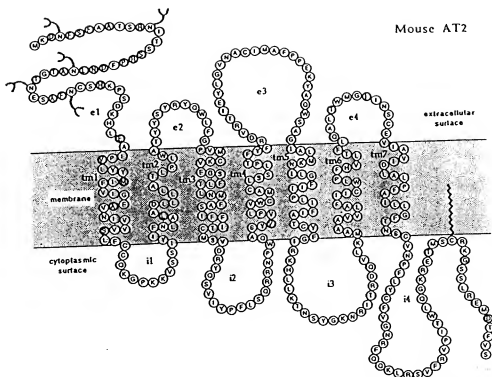


Figure 2. Putative organization of the murine AT₂ receptor within the cell membrane. Five potential N-linked glycosylated sites in the amino-terminus and a putative palmitoylated cysteine in the carboxy-terminus are indicated. Residues which differ between mouse and human AT₂ sequences are in bold.

The deduced amino-acid sequence of the mouse AT₂ polypeptide (Fig. 2) displays all the features characteristic of G-protein coupled receptors (24), including the presence of seven membrane-spanning domains, potential N-linked glycosylation sites, and consensus sites for phosphorylation by protein kinases in the carboxy terminal tail. Sequence comparison indicates high conservation (92% amino-acid sequence homology) between human and murine AT₂ receptors (Fig. 2). Most divergent residues are clustered in the extracellular, N-terminal portion of the molecule. Despite these differences, five putative N-linked glycosylation sites are conserved at identical positions in the amino-terminal part of the AT₂ receptor in both species.

The isolated cDNA clones were functionally expressed in COS cells, yielding pharmacological parameters identical to those of endogenous Ang II receptors from N1E-115 cells (Table I). This confirms that N1E-115 cells express exclusively the AT₂ receptor subtype, and identifies this receptor entity at the molecular level.

FUNCTIONAL STUDIES OF AT₂ RECEPTORS: COUPLING TO PROTEIN TYROSINE DEPHOSPHORYLATION

The pharmacological and molecular identification of a single class of AT₂ receptors in N1E-115 cells prompted us to further analyze this receptor subtype at the functional level.

The relationship between AT₂ signaling and intracellular protein tyrosine phosphorylation was investigated by Western blot analysis of total cell lysates using anti-phosphotyrosine antibodies. Treatment of NIE-115 cells with Ang II led to a reduction in tyrosine phosphorylation of several endogenous proteins, of apparent molecular masses 80, 97, 120, 150 and 180 kDa, respectively (Fig. 3). This effect was rapid and transient, showing a maximum at 5 to 10 minutes and declining at 30 minutes. This response could be blocked in the presence of an excess of the antagonist (Sar, Ile)-Ang II and was also obtained following treatment with the AT₂-selective agonist CGP 42112 (9).

Preliminary studies of Chinese Hamster Ovary (CHO) cells stably expressing the mouse AT₂ receptor indicated that in this cellular model, AT₂ stimulation also results in the transient dephosphorylation of cellular proteins on tyrosine residues (data not shown). The same intracellular effect could not however be detected in COS cells transiently transfected with either the murine or the human AT₂ receptor cDNA. This may indicate that COS cells lack one or several components of the AT₂ signaling pathway. It is worth noting that other groups reporting the cloning of AT₂ receptor cDNA failed to detect any intracellular effect of this receptor after both transient (16) and stable (6) overexpression in COS cells.

Our results showing a reduction of intracellular protein tyrosine phosphorylation may be explained by stimulation of a protein tyrosine phosphatase (PTP) activity, or by inhibition of a tyrosine kinase-mediated pathway. Given the complexity of intracellular cascades of kinases and phosphatases (25-27), it is indeed possible that AT₂ receptors differentially affect several types of enzymes acting at different levels. In the PC12W cell line, AT₂ receptors have been reported to stimulate a PTP activity by a G-protein-independent mechanism (4, 7) and to inhibit a PTP through a pertussis toxin-mediated pathway (6, 8). Progress in understanding AT₂ signaling will greatly benefit from investigating molecular determinants of AT₂-mediated pathway, including identification of the G-protein(s) involved, search for cellular "interaction partners" of the receptor, elucidation of PTPs/TKs activities modulated by the AT₂ subtype, and identification of endogenous phosphoproteins which are dephosphorylated following AT₂ receptor activation.

The relationship between AT₂ receptors and tyrosine phosphorylation suggests a link with physiological processes such as cell growth, differentiation or cellular adhesion, which are known to involve cascades of protein kinases and phosphatases (28). Possible role for AT₂ receptors in these processes is also consistent with increased levels of expression of this subtype during embryonic development (29) and in pathological situations such as vascular growth (30) and tissue repair (31). *In vivo* studies have indeed identified AT₂ receptors as

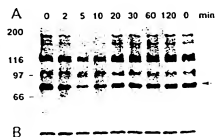


Figure 3. Ang II-induced tyrosine dephosphorylation in NIE-115 cells. (A) NIE-115 cells were incubated with Ang II for various periods of time, before lysis and subsequent immunoblotting with anti-phosphotyrosine antibodies. On the left are indicated the positions of standard molecular weight markers, and the arrow on the right shows the position of a major phosphoprotein of 80 kDa. (B) After removal of bound antibodies, the same blot was incubated with anti-zinnin antibodies for internal control of the amount of proteins in each lane.

participating in vascular neointima formation (30) and angiogenesis (32). More recently, anti-proliferative effects of AT₂ receptors in primary cultures of rat endothelial coronary cells have been demonstrated (33). Other studies performed in cultured cells tend to indicate, however, inverse relationship between expression of AT₂ receptors and cell proliferation (34-36).

ARE THERE MULTIPLE SUBCLASSES OF AT₂ RECEPTORS?

Studies conducted in the rat brain have pointed out possible heterogeneity of AT₂ receptors. Quantitative autoradiography revealed differential sensitivity of CGP 42112-binding sites to analogs of GTP (37) and to reducing reagents (38, 39). In addition, immunohistochemical staining of rat brain sections using anti-AT₂ polyclonal antibodies correlated with, but was not identical to patterns of radioligand binding (40). As mentioned before, two pharmacologically, biochemically and immunologically distinct populations of AT₂ receptors could be identified in NIE-115 cells (22). These observations, as well as the diverse and sometimes contradictory reports of AT₂ signaling, may be due to expression of a single molecular entity in different cell environments, and/or may reflect the existence of multiple subclasses of AT₂ receptors.

The AT₂ receptor gene exists as a single copy in the genome of both human (18) and mouse (17). This argues against the possibility that several highly homologous AT₂ genes exist, as is the case for rodent AT_{1a} and AT_{1b}. In addition, the coding region of the AT₂ gene is devoid of introns, thus ruling out the hypothesis that alternative splicing may lead to multiple forms of receptors. It remains possible however that "AT₂ receptors" in fact consist of several molecular entities, that do not share high amino-acid sequence homology but that all exhibit high affinity for ligands such as CGP 42112 and PD 123319 which define the AT₂ subtype pharmacologically.

Further characterization of AT₂ receptors in a variety of cellular environments is now made possible by the development of series of new ligands (41, 42) and may help discriminate between putative AT₂ receptor subtypes. The molecular basis of AT₂ receptor heterogeneity will also certainly be investigated in the coming years, as more and more AT₂ cDNA clones will be isolated from different tissues and cell lines.

ACKNOWLEDGMENTS

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42. Chang, L. L. et al. (1994). *J Med Chem* 37: 4464-4478.

Human: (a) a c c a u t d i p o t (v a n
page 3)



Protein

ANNEX II

(9 pages)

1: P50052. Type-2 angiotensi...[gi:1703214]

BLink, Domains, Links

LOCUS P50052 363 aa linear PRI 15-JUL-1998

DEFINITION TYPE-2 ANGIOTENSIN II RECEPTOR (AT2).

ACCESSION P50052

VERSION P50052 GI:1703214

SOURCE swissprot: locus AG22_HUMAN, accession P50052;

class: standard.

extra accessions: Q13016, created: Oct 1, 1996.

sequence updated: Oct 1, 1996.

annotation updated: Jul 15, 1998.

xrefs: gi: 747969, gi: 747970, gi: 510700, gi: 510701, gi: 607811,

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1143833, gi: 1143834, gi: 860958, gi: 860959

xrefs (non-sequence databases): GCRDBGCR_1057, GCRDBGCR_1245,

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GCRDBGCR_2056, MIM 300034, PROSITEPS00237

G-PROTEIN COUPLED RECEPTOR; TRANSMEMBRANE; GLYCOPROTEIN.

KEYWORDS

SOURCE Homo sapiens (human)

ORGANISM Homo sapiens

Eukaryota; Metazoa; Chordata; Vertebrata; Mammalia; Eutheria;

Primates; Catarrhini; Hominidae; Homo.

REFERENCE 1 (residues 1 to 363)

AUTHORS Martin, M.M. and Elton, T.S.

TITLE The sequence and genomic organization of the human type 2

angiotensin II receptor

JOURNAL Biochem. Biophys. Res. Commun. 209 (2), 554-562 (1995)

MEDLINE 95251653

REMARK SEQUENCE FROM N.A.

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REFERENCE 2 (residues 1 to 363)

AUTHORS Chassagne, C., Beatty, B.G. and Meloche, S.

TITLE Assignment of the human angiotensin II type 2 receptor gene (AGTR2)

to chromosome Xq22-q23 by fluorescence in situ hybridization

JOURNAL Genomics 25 (2), 601-603 (1995)

MEDLINE 95309939

REMARK SEQUENCE FROM N.A.

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REFERENCE 3 (residues 1 to 363)

AUTHORS Koike, G., Horiuchi, M., Yamada, T., Szpirer, C., Jacob, H.J. and

Dzau, V.J.

TITLE Human type 2 angiotensin II receptor gene: cloned, mapped to the X

chromosome, and its mRNA is expressed in the human lung

JOURNAL Biochem. Biophys. Res. Commun. 203 (3), 1842-1850 (1994)

MEDLINE 95032069

REMARK SEQUENCE FROM N.A.

TISSUE=BLOOD

REFERENCE 4 (residues 1 to 363)

AUTHORS Tsuzuki, S., Ichiki, T., Nakakubo, H., Kitami, Y., Guo, D.F., Shirai, H.

and Inagami, T.

TITLE Molecular cloning and expression of the gene encoding human

angiotensin II type 2 receptor

JOURNAL Biochem. Biophys. Res. Commun. 200 (3), 1449-1454 (1994)

MEDLINE 94242007

REMARK SEQUENCE FROM N.A.

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REFERENCE 5 (residues 1 to 363)
 AUTHORS Martin, M.M., Su, B. and Elton, T.S.
 TITLE Molecular cloning of the human angiotensin II type 2 receptor cDNA
 JOURNAL Biochem. Biophys. Res. Commun. 205 (1), 645-651 (1994)
 MEDLINE 95091796
 REMARK SEQUENCE FROM N.A.
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REFERENCE 6 (residues 1 to 363)
 AUTHORS Lazard, D., Briand-Sutren, M.M., Villageois, P., Mattei, M.G., Strosberg, A.D. and Nahmias, C.
 TITLE Molecular characterization and chromosome localization of a human angiotensin II AT2 receptor gene highly expressed in fetal tissues
 JOURNAL Recept. Channels 2 (4), 271-280 (1994)
 MEDLINE 95236034
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REFERENCE 7 (residues 1 to 363)
 AUTHORS KATSUYA, T. and DZAU, V.J.
 TITLE Direct Submission
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REFERENCE 8 (residues 1 to 363)
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 TITLE Direct Submission
 JOURNAL Submitted (~JUN-1995) TO EMBL/GENBANK/DBJ DATA BANKS
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 TISSUE=UTERUS

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 [SUBCELLULAR LOCATION] INTEGRAL MEMBRANE PROTEIN.
 [TISSUE SPECIFICITY] IN ADULT, HIGHLY EXPRESSED IN MYOMETRIUM WITH LOWER LEVELS IN ADRENAL GLAND AND FALLOPIAN TUBE. VERY HIGHLY EXPRESSED IN FETAL KIDNEY AND INTESTINE.
 [SIMILARITY] BELONGS TO FAMILY 1 OF G-PROTEIN COUPLED RECEPTORS.

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/note="CW -> WC (IN REF. 5)."

Region 272
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Region 314..363
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Answer
II

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IGIN
1 mkgnstlatt sknitsglhf glvnisgnne stlnscqkps dkhldaipil yyiifvigfl
61 vnivvvtlfc cqkgpkkvss iyifnlavad llllatlplw atyysrydw lfgpvmckvf
121 gsfltlmfa siffitcmv dryqsviypf lsqrrnpwqa syivplvwcm aclsslptfy
181 frdvrtieyl gvnacimafp pekyaqwsag ialmknilgf iiplifiatc yfgirkhlk
241 tnsyqknrit rdqvlkmaa vvlafiicwl pfhvltflda lawmgvinsc eviavidlal
301 pfaillgftn scvnpflycf vgnrfqqklr svfrvpitwl qgkresmscr kssslremet
361 fvs

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Sequence Revision History

PubMed Nucleotide Protein Genome Structure PMC Taxonomy OMIM Books

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Revision history for P50052

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Entrez: Upload a
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protein or nucleotide
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How to create WWW
links to Entrez

Out

bbby

Related resources

AST

Reference sequence project

Link

Members of orthologous groups

Entrez reviews on the web

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Accession P50052 was first seen at NCBI on Dec 3 1996 9:38 PM



1: P50052. Type-2 angiotensi...[gi:1703214]

BLink. Domains, Links

>CDS P50052 363 aa linear PRI 15-MAR-2004
 >DEFINITION Type-2 angiotensin II receptor (AT2).
 >ACCESSION P50052
 >VERSION P50052 GI:1703214
 >SOURCE swissprot: locus AG22_HUMAN, accession P50052;
 class: standard.

extra accessions: Q13016, created: Oct 1, 1996.

sequence updated: Oct 1, 1996.

annotation updated: Mar 15, 2004.

xrefs: gi: 747969, gi: 747970, gi: 510700, gi: 510701, gi: 607811,

gi: 607812, gi: 558882, gi: 558883, gi: 595934, gi: 595935, gi:

32482004, gi: 32482005, gi: 1143833, gi: 1143834, gi: 860958, gi:

860959, gi: 1082208

xrefs (non-sequence databases): HSSPP34996, GenewHGNC:338, MIM

300034, GO0005887, GO0004945, GO0004860, GO0006915, GO0007610,

GO0007166, GO0008217, InterProIPR000276, PfamPF00001,

PRINTSPR00237, PROSITEPS00237, PROSITEPS0262

>KEYWORDS G-protein coupled receptor; Transmembrane; Glycoprotein;
 Polymorphism.

>ORIGIN Homo sapiens (human)

>ORGANISM Homo sapiens

Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;

Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.

1 (residues 1 to 363)

>REFERENCE Martin, M.M. and Elton, T.S.

>AUTHORS The sequence and genomic organization of the human type 2
 >TITLE angiotensin II receptor

>JOURNAL Biochem. Biophys. Res. Commun. 209 (2), 554-562 (1995)

>MEDLINE 95251653

>PUBMED 7733925

>REMARK SEQUENCE FROM N.A.

TISSUE=Liver

>REFERENCE 2 (residues 1 to 363)

>AUTHORS Chassagne, C., Beatty, B.G. and Meloche, S.

>TITLE Assignment of the human angiotensin II type 2 receptor gene (AGTR2)
 to chromosome Xq22-q23 by fluorescence in situ hybridization

>JOURNAL Genomics 25 (2), 601-603 (1995)

>MEDLINE 95309939

>PUBMED 7790004

>REMARK SEQUENCE FROM N.A.

TISSUE=Placenta

>REFERENCE 3 (residues 1 to 363)

>AUTHORS Koike, G., Horiuchi, M., Yamada, T., Szpirer, C., Jacob, H.J. and
 Dzau, V.J.

>TITLE Human type 2 angiotensin II receptor gene: cloned, mapped to the X
 chromosome, and its mRNA is expressed in the human lung

>JOURNAL Biochem. Biophys. Res. Commun. 203 (3), 1842-1850 (1994)

>MEDLINE 95032069

>PUBMED 7945336

>REMARK SEQUENCE FROM N.A.

TISSUE=Blood

>REFERENCE 4 (residues 1 to 363)

>AUTHORS Tsuzuki, S., Ichiki, T., Nakakubo, H., Kitami, Y., Guo, D.F., Shirai, H.

and Inagami, T.
TITLE Molecular cloning and expression of the gene encoding human
angiotensin II type 2 receptor
JOURNAL Biochem. Biophys. Res. Commun. 200 (3), 1449-1454 (1994)
MEDLINE 94242007
PUBMED 8185599
REMARK SEQUENCE FROM N.A.
TISSUE=Placenta
REFERENCE 5 (residues 1 to 363)
AUTHORS Martin, M.M., Su, B. and Elton, T.S.
TITLE Molecular cloning of the human angiotensin II type 2 receptor cDNA
JOURNAL Biochem. Biophys. Res. Commun. 205 (1), 645-651 (1994)
MEDLINE 95091796
PUBMED 7999093
REMARK SEQUENCE FROM N.A.
TISSUE=Lung
REFERENCE 6 (residues 1 to 363)
AUTHORS Lazard, D., Briand-Sutren, M.M., Villageois, P., Mattei, M.G.,
Strosberg, A.D. and Nahmias, C.
TITLE Molecular characterization and chromosome localization of a human
angiotensin II AT2 receptor gene highly expressed in fetal tissues
JOURNAL Recept. Channels 2 (4), 271-280 (1994)
MEDLINE 95236034
PUBMED 7719706
REMARK SEQUENCE FROM N.A.
TISSUE=Placenta
REFERENCE 7 (residues 1 to 363)
AUTHORS Kopatz, S.A., Aronstam, R.S. and Sharma, S.V.
TITLE Direct Submission
JOURNAL Submitted (~JUN-2003)
REMARK SEQUENCE FROM N.A.
REFERENCE 8 (residues 1 to 363)
AUTHORS Katsuya, T. and Dzau, V.J.
TITLE Direct Submission
JOURNAL Submitted (~JAN-1996)
REMARK SEQUENCE OF 1-22 FROM N.A.
TISSUE=Blood
REFERENCE 9 (residues 1 to 363)
AUTHORS Warnecke, C.H., Holzmeister, J., Regitz-Zagrosek, V. and Fleck, E.
TITLE Direct Submission
JOURNAL Submitted (~JUN-1995)
REMARK SEQUENCE OF 1-16 FROM N.A.
TISSUE=Uterus

COMMENT

This SWISS-PROT entry is copyright. It is produced through a
collaboration between the Swiss Institute of Bioinformatics and
the EMBL outstation - the European Bioinformatics Institute.
The original entry is available from <http://www.expasy.ch/sprot>
and <http://www.ebi.ac.uk/sprot>

[FUNCTION] Receptor for angiotensin II. May have a role in cell
morphogenesis and related events in growth and development.
[SUBCELLULAR LOCATION] Integral membrane protein.
[TISSUE SPECIFICITY] In adult, highly expressed in myometrium with
lower levels in adrenal gland and fallopian tube. Very highly
expressed in fetal kidney and intestine.
[SIMILARITY] Belongs to family 1 of G-protein coupled receptors.

FEATURES
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/organism="Homo sapiens"
/db_xref="taxon:9606"
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Protein 1..363

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/note="N-linked (GlcNAc...) (Potential)."
Site 13
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/gene="AGTR2"
/region_name="Domain"
/note="Cytoplasmic (Potential)."
Region 81..102
/gene="AGTR2"
/region_name="Transmembrane region"
/note="2 (Potential)."
Region 103..119
/gene="AGTR2"
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/note="Extracellular (Potential)."
Region 120..140
/gene="AGTR2"
/region_name="Transmembrane region"
/note="3 (Potential)."
Region 141..160
/gene="AGTR2"
/region_name="Domain"
/note="Cytoplasmic (Potential)."
Region 161..179
/gene="AGTR2"
/region_name="Transmembrane region"
/note="4 (Potential)."
Region 180..208
/gene="AGTR2"
/region_name="Domain"
/note="Extracellular (Potential)."
Region 209..234
/gene="AGTR2"
/region_name="Transmembrane region"
/note="5 (Potential)."
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/gene="AGTR2"

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/region_name="Transmembrane region"
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Region 272
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/region_name="7 (Potential)."
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181 frdrtieyl gvnacimafp pekyaqwsag ialmknlgf iiplifiatc yfgirkhlk
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301 pfaillgftn scvnpflycf vgnrfqgklr svfrvpitwl qgkresmscr kssslremet
361 fvs

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ANNEX III

(8 pages)

1: P35374. Type-2 angiotensi...[gi:543779]

BLink. Domains. Links

NCUS P35374 363 aa linear ROD 15-JUL-1998
 :FINITION TYPE-2 ANGIOTENSIN II RECEPTOR (AT2).
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 :RSION P35374 GI:543779
 :SOURCE swissprot: locus AG22_MOUSE, accession P35374;
 class: standard.
 created: Jun 1, 1994.
 sequence updated: Jun 1, 1994.
 annotation updated: Jul 15, 1998.
 xrefs: gi: 455900, gi: 455901, gi: 439862, gi: 439863, gi: 443584,
 gi: 474274, gi: 487848, , gi: 607834, , gi: 543186
 xrefs (non-sequence databases): GCRDBGCR_0890, GCRDBGCR_1007,
 GCRDBGCR_1010, MGI87966, PROSITEPS00237
 :YWORDS G-PROTEIN COUPLED RECEPTOR; TRANSMEMBRANE; GLYCOPROTEIN;
 PHOSPHORYLATION.
 :URCE Mus musculus (house mouse)
 ORGANISM Mus musculus
 Eukaryota; Metazoa; Chordata; Vertebrata; Mammalia; Eutheria;
 Rodentia; Sciurognathi; Muridae; Murinae; Mus.
 :REFERENCE 1 (residues 1 to 363)
 AUTHORS Nakajima,M., Mukoyama,M., Pratt,R.E., Horiuchi,M. and Dzau,V.J.
 TITLE Cloning of cDNA and analysis of the gene for mouse angiotensin II
 type 2 receptor
 JOURNAL Biochem. Biophys. Res. Commun. 197 (2), 393-399 (1993)
 MEDLINE 94092107
 REMARK SEQUENCE FROM N.A.
 STRAIN=BALB/C; TISSUE=FETAL
 :REFERENCE 2 (residues 1 to 363)
 AUTHORS Ichiki,T., Herold,C.L., Kambayashi,Y., Bardhan,S. and Inagami,T.
 TITLE Cloning of the cDNA and the genomic DNA of the mouse angiotensin II
 type 2 receptor
 JOURNAL Biochim. Biophys. Acta 1189 (2), 247-250 (1994)
 MEDLINE 94122216
 REMARK SEQUENCE FROM N.A.
 STRAIN=BALB/C
 :REFERENCE 3 (residues 1 to 363)
 AUTHORS Nahmias,C., Cazaubon,S.M., Sutren,M., Masson,M., Lazard,D.,
 Villageois,P., Elbaz,N. and Strosberg,A.D.
 TITLE Molecular and functional characterization of angiotensin II AT2
 receptor in neuroblastoma N1E-115 cells
 JOURNAL Adv. Exp. Med. Biol. 396, 167-173 (1996)
 MEDLINE 96337434
 REMARK SEQUENCE FROM N.A.
 :REFERENCE 4 (residues 1 to 363)
 AUTHORS Horiuchi,M., Koike,G., Yamada,T., Mukoyama,M., Nakajima,M. and
 Dzau,V.J.
 TITLE The growth-dependent expression of angiotensin II type 2 receptor
 is regulated by transcription factors interferon regulatory
 factor-1 and -2
 JOURNAL J. Biol. Chem. 270 (34), 20225-20230 (1995)
 MEDLINE 95378283
 REMARK SEQUENCE FROM N.A.
 STRAIN=BALB/C; TISSUE=LIVER

Answer
III

COMMENT [FUNCTION] RECEPTOR FOR ANGIOTENSIN II. MAY HAVE A ROLE IN CELL MORPHOGENESIS AND RELATED EVENTS IN GROWTH AND DEVELOPMENT.
[SUBCELLULAR LOCATION] INTEGRAL MEMBRANE PROTEIN.
[TISSUE SPECIFICITY] ABUNDANT IN FETUS, LOWER LEVELS IN ADULT BRAIN.
[PTM] CARBOXYL-TERMINAL SER OR THR RESIDUES MAY BE PHOSPHORYLATED.
[SIMILARITY] BELONGS TO FAMILY 1 OF G-PROTEIN COUPLED RECEPTORS.

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Protein	1..363 /gene="AGTR2" /product="TYPE-2 ANGIOTENSIN II RECEPTOR"
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Site	13 /gene="AGTR2" /site_type="glycosylation"
Site	24 /gene="AGTR2" /site_type="glycosylation"
Site	29 /gene="AGTR2" /site_type="glycosylation"
Site	34 /gene="AGTR2" /site_type="glycosylation"
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Region	81..102 /gene="AGTR2" /region_name="Transmembrane region" /note="2."
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Answer


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/ region_name="Transmembrane region"
/ note="7."
Region 314..363
/ gene="AGTR2"
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IGIN

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121 gsfltnmfia siffitcmsv dryqsviypf lsqrnpwqa syvvpwvcm acfsslpfty
181 frdvrtieyl gvnacimafp pekyaqwsag ialmknifgf iiplifiatc yfgirkhlhk
241 tnsygnrit rdqvlkmaa vvlafiiawl pfhylvfla ltwmgiinsc eviavidlal
301 pfaillgftn scvnpflycf vgnfrqqklr svfrvpitwl qgkretmscr kgsslremdt
361 fvs
```

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Sous-Hashtag

Annexe
III

Sequence Revision History

Published Nucleotide Protein Genome Structure PMC Taxonomy OMIM Books

Find (Accessions, GI numbers or FastA style SeqIds) P35374

Show difference between I and II as GenBank/GenPept

Revision history for P35374

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Accession P35374 was first seen at NCBI on Sep 22 1994 4:36 PM



Entrez PubMed Nucleotide Protein Genome Structure PMC Taxonomy Books
 Search **Protein** for **Go** **Clear**
 Limits Preview/Index History Clipboard Details
 Display **default** Show: **20** Send to **File** **Get Subsequence** **Features**

1: P35374. Type-2 angiotensi...[gi:543779]

BLink, Domains, Links

DCUS P35374 363 aa linear ROD 15-MAR-2004
 DEFINITION Type-2 angiotensin II receptor (AT2).
 CESSION P35374
 ERSION P35374 GI:543779
 SOURCE swissprot: locus AG22_MOUSE, accession P35374;
 class: standard.
 created: Jun 1, 1994.
 sequence updated: Jun 1, 1994.
 annotation updated: Mar 15, 2004.
 xrefs: gi: 455900, gi: 455901, gi: 439862, gi: 439863, gi: 443584,
 gi: 474274, gi: 487848, gi: 1881537, gi: 607834, gi: 607836, gi:
 13277866, gi: 13277867, gi: 2137148
 xrefs (non-sequence databases): HSSPP34996, MGI87966,
 InterProIPR000276, PfamPF00001, PRINTSPR00237, PROSITEPS00237,
 PROSITEPS0262
 YWORDS G-protein coupled receptor; Transmembrane; Glycoprotein;
 Phosphorylation.
 URCE Mus musculus (house mouse)
 ORGANISM Mus musculus
 Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
 Mammalia; Eutheria; Rodentia; Sciurognathi; Muridae; Murinae; Mus.
 REFERENCE 1 (residues 1 to 363)
 AUTHORS Nakajima, M., Mukoyama, M., Pratt, R.E., Horiuchi, M. and Dzau, V.J.
 TITLE Cloning of cDNA and analysis of the gene for mouse angiotensin II
 type 2 receptor
 JOURNAL Biochem. Biophys. Res. Commun. 197 (2), 393-399 (1993)
 MEDLINE 94092107
 PUBMED 8267573
 REMARK SEQUENCE FROM N.A.
 STRAIN=BALB/c; TISSUE=Fetal
 2 (residues 1 to 363)
 REFERENCE 2 (residues 1 to 363)
 AUTHORS Ichiki, T., Herold, C.L., Kambayashi, Y., Bardhan, S. and Inagami, T.
 TITLE Cloning of the cDNA and the genomic DNA of the mouse angiotensin II
 type 2 receptor
 JOURNAL Biochim. Biophys. Acta 1189 (2), 247-250 (1994)
 MEDLINE 94122216
 PUBMED 8292631
 REMARK SEQUENCE FROM N.A.
 STRAIN=BALB/c
 3 (residues 1 to 363)
 REFERENCE 3 (residues 1 to 363)
 AUTHORS Nahmias, C., Cazaubon, S.M., Sutren, M., Masson, M., Lazard, D.,
 Villageois, P., Elbaz, N. and Strosberg, A.D.
 TITLE Molecular and functional characterization of angiotensin II AT2
 receptor in neuroblastoma N1E-115 cells
 JOURNAL Adv. Exp. Med. Biol. 396, 167-173 (1996)
 MEDLINE 96337434
 PUBMED 8726696
 REMARK SEQUENCE FROM N.A.
 4 (residues 1 to 363)
 REFERENCE 4 (residues 1 to 363)
 AUTHORS Horiuchi, M., Koike, G., Yamada, T., Mukoyama, M., Nakajima, M. and
 Dzau, V.J.
 TITLE The growth-dependent expression of angiotensin II type 2 receptor
 is regulated by transcription factors interferon regulatory

A-1180

TII

factor-1 and -2
 J. Biol. Chem. 270 (34), 20225-20230 (1995)
 MEDLINE 95378283
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 REMARK SEQUENCE FROM N.A.
 STRAIN=BALB/c; TISSUE=Liver
 REFERENCE 5 (residues 1 to 363)
 AUTHORS Strausberg,R.L., Feingold,E.A., Grouse,L.H., Derge,J.G.,
 Klausner,R.D., Collins,F.S., Wagner,L., Shenmen,C.M., Schuler,G.D.,
 Altschul,S.F., Zeeberg,B., Buetow,K.H., Schaefer,C.F., Bhat,N.K.,
 Hopkins,R.F., Jordan,H., Moore,T., Max,S.I., Wang,J., Hsieh,F.,
 Diatchenko,L., Marusina,K., Farmer,A.A., Rubin,G.M., Hong,L.,
 Stapleton,M., Soares,M.B., Bonaldo,M.F., Casavant,T.L.,
 Scheetz,T.E., Brownstein,M.J., Usdin,T.B., Toshiyuki,S.,
 Carninci,P., Prange,C., Raha,S.S., Loquellano,N.A., Peters,G.J.,
 Abramson,R.D., Mullahy,S.J., Bosak,S.A., McEwan,P.J.,
 McKernan,K.J., Malek,J.A., Gunaratne,P.H., Richards,S.,
 Worley,K.C., Hale,S., Garcia,A.M., Gay,L.J., Hulyk,S.W.,
 Villalón,D.K., Muzny,D.M., Sodergren,E.J., Lu,X., Gibbs,R.A.,
 Fahey,J., Helton,E., Kettman,M., Madan,A., Rodrigues,S.,
 Sanchez,A., Whiting,M., Madan,A., Young,A.C., Shevchenko,Y.,
 Bouffard,G.G., Blakesley,R.W., Touchman,J.W., Green,E.D.,
 Dickson,M.C., Rodriguez,A.C., Grimwood,J., Schmutz,J., Myers,R.M.,
 Butterfield,Y.S.N., Krzywinski,M.I., Skalska,U., Smailus,D.E.,
 Schnerch,A., Schein,J.E., Jones,S.J.M. and Marra,M.A.
 TITLE Generation and initial analysis of more than 15,000 full-length
 human and mouse cDNA sequences
 JOURNAL Proc. Natl. Acad. Sci. U.S.A. 99 (26), 16899-16903 (2002)
 MEDLINE 22388257
 PUBMED 12477932
 REMARK SEQUENCE FROM N.A.
 STRAIN=C57BL/6J; TISSUE=Mammary gland

COMMENT

 This SWISS-PROT entry is copyright. It is produced through a
 collaboration between the Swiss Institute of Bioinformatics and
 the EMBL outstation - the European Bioinformatics Institute.
 The original entry is available from <http://www.expasy.ch/sprot>
 and <http://www.ebi.ac.uk/sprot>

[FUNCTION] Receptor for angiotensin II. May have a role in cell
 morphogenesis and related events in growth and development.
 [SUBCELLULAR LOCATION] Integral membrane protein.
 [TISSUE SPECIFICITY] Abundant in fetus, lower levels in adult
 brain.
 [PTM] Carboxyl-terminal Ser or Thr residues may be phosphorylated.
 [SIMILARITY] Belongs to family 1 of G-protein coupled receptors.

FEATURES Location/Qualifiers
 source 1..363
 /organism="Mus musculus"
 /db_xref="taxon:10090"
 gene 1..363
 /gene="AGTR2"
 Protein 1..363
 /gene="AGTR2"
 /product="Type-2 angiotensin II receptor"
 Region 1..45
 /gene="AGTR2"
 /region_name="Domain"
 /note="Extracellular (Potential)."
 Site 4
 /gene="AGTR2"
 /site_type="glycosylation"
 /note="N-linked (GlcNAc...) (Potential)."
 Site 13

Anusa

III

Site /gene="AGTR2"
/site_type="glycosylation"
/note="N-linked (GlcNAc...) (Potential)."
24

Site /gene="AGTR2"
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29

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/note="N-linked (GlcNAc...) (Potential)."
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46..71

Region /gene="AGTR2"
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72..80

Region /gene="AGTR2"
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/note="2 (Potential)."
81..102

Region /gene="AGTR2"
/region_name="Domain"
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103..119

Region /gene="AGTR2"
/region_name="Transmembrane region"
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120..140

Region /gene="AGTR2"
/region_name="Domain"
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141..160

Region /gene="AGTR2"
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161..179

Region /gene="AGTR2"
/region_name="Domain"
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180..208

Region /gene="AGTR2"
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209..234

Region /gene="AGTR2"
/region_name="Domain"
/note="Cytoplasmic (Potential)."
235..256

Region /gene="AGTR2"
/region_name="Transmembrane region"
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257..278

Region /gene="AGTR2"
/region_name="Domain"
/note="Extracellular (Potential)."
279..285

Region /gene="AGTR2"
/region_name="Domain"
/note="Extracellular (Potential)."
286..313

/gene="AGTR2"
 /region_name="Transmembrane region"
 /note="7 (Potential)."
 314..363
 /gene="AGTR2"
 /region_name="Domain"
 /note="Cytoplasmic (Potential)."
 354
 /gene="AGTR2"
 /site_type="phosphorylation"
 /note="(BY PKC) (POTENTIAL)."
 /

Answer

III

RIGIN
 1 mkdndsfaat srnitssrpf dnlnatgtne safncshkps dkhleaipvl yymifvigfa
 61 vniuvvslfc cqkgpkkvss iyifnlalad llllatlplw atyysyrydw lfgpvmckvf
 121 gsfltlmfa siffitcmv dryqsviypf lsqrnpwqa syvvpvlwcm aclsslptfy
 181 frdvrtieyl gvnacimafp pekyaqwsag ialmknlgf iiplifiatc yfgirkhllk
 241 tnsygknrit rdqvlkmaaa vvlafiicwl pfhvlftlda ltwmgiinsc eviavidlal
 301 pfaillgftn scvnpflycf vgnrfqqklr svfrvpitwl qgkretmscr kgsslremdt
 361 fvs

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☐ 1: B652532. UI-M-AM0-adp-b-06...[gi:9978333]

Links

IDENTIFIERS

dbEST Id: 5909367
EST name: UI-M-AM0-adp-b-06-0-UI.r1
GenBank Acc: BE652532
GenBank gi: 9978333

CLONE INFO

Clone Id: UI-M-AM0-adp-b-06-0-UI (5')
Source: The NIH - University of Iowa Brain Molecular Anatomy
Project: NIH-Iowa BMAP (Bento Soares, Thomas Casavar
Val Sheffield)
Id as DNA: UI-M-AM0-adp-b-06-0-UI
Id in host: UI-M-AM0-adp-b-06.r1
DNA type: cDNA

PRIMERS

Sequencing: M13 Reverse
PolyA Tail: Unknown

SEQUENCE

GCTGCTTCAAGCGCCTGTGAGAAGCTAGAAAAGGCTAGGACTGACTTACAG/
CAAGAATTTGTCCAGAACTAAACCAGCAGCATCAGACAGACCGGACCGGAAC
CGGCTGAAGGACTTATACACCGCAGAGTGTGAGAAGCTTCAGAGCATTTAC/
GCAGAAAAATATAAACTCAACTGCAAGAGCAGTTTGACAACCTAAACGCC
ACCACTAAGCTTGAGATTGAAGCTAGCCACTCGGAGAAGGTGGAATTGCTG/
TATGAAACCTCCCTTTAGAAATCAAGAAGGCCATGAGATGGAGAAGAAG/
GATCTGCTTAATGAGAAGCAGGAATCGCTGGAGAAACAAATCAATGATCTG/
AACGATGCTTTAAACGAAAGGTTGAAATCAGAGGAGCAAAGCAACTGTCA/
GCGAATTCAAAAACCTCAGGTCATGTATCTGGAGC

Entry Created: Sep 6 2000
Last Updated: Sep 6 2000

COMMENTS

cDNA Library Preparation: M.B. Soares Lab Clone
distribution: Researchers may obtain BMAP cDNA clone
RESEARCH GENETICS. It should be noted that Bento Soa
generating a small number of additional specialized
non-redundant arrays of BMAP cDNAs whose availabilit
be considered under appropriate and limited collabor
arrangements

LIBRARY

Lib Name: NIH BMAP MAM

Strain: C57BL/6J
Develop. stage: 27-32 days
Lab host: DH10B (Life Technologies)
Vector: pT7T3D-Pac (Pharmacia) with a modified polylinker
R. Site 1: Not I
R. Site 2: Eco RI
Description: The NIH_BMAP_MAM library is a non-normalized library constructed from mouse amygdala. The tag is a string of nucleotides present between the Not I site and the Eco RI site. The library was constructed as described by Lennon and Soares, Genome Research 6: 791-806, 1996. The library was provided by Ms. Annie Novakovich, Zivic-Miller Laboratory.

SUBMITTER

Name: Chin, H
Institution: National Institute of Mental Health
Address: 6001 Executive Blvd. Room 7N-7190, MSC 9643, Bethesda, MD 20892-9643, USA
Tel: 301 443 1706
Fax: 301 443 9890
E-mail: mEST@mail.nih.gov

CITATIONS

Medline UID: 97044477
Title: Normalization and subtraction: two approaches to facilitate gene discovery
Authors: Bonaldo, M.F., Lennon, G., Soares, M.B.
Citation: Genome Res. 6 (9): 791-806 1996

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GI	Version	Update Date	Status
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LocusLink provides curated information for human, fruit fly, mouse, rat, and zebrafish

Accession BE652532 was first seen at NCBI on Sep 6 2000 6:24 PM

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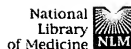
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1: Genome Res. 1996 Sep;6(9):791-806.

Related Articles, Links

Normalization and subtraction: two approaches to facilitate gene discovery.

Bonaldo MF, Lennon G, Soares MB.

Department of Psychiatry, College of Physicians and Surgeons of Columbia University, New York, New York, USA.

Large-scale sequencing of cDNAs randomly picked from libraries has proven to be a very powerful approach to discover (putatively) expressed sequences that, in turn, once mapped, may greatly expedite the process involved in the identification and cloning of human disease genes. However, the integrity of the data and the pace at which novel sequences can be identified depends to a great extent on the cDNA libraries that are used. Because altogether, in a typical cell, the mRNAs of the prevalent and intermediate frequency classes comprise as much as 50-65% of the total mRNA mass, but represent no more than 1000-2000 different mRNAs, redundant identification of mRNAs of these two frequency classes is destined to become overwhelming relatively early in any such random gene discovery programs, thus seriously compromising their cost-effectiveness. With the goal of facilitating such efforts, previously we developed a method to construct directionally cloned normalized cDNA libraries and applied it to generate infant brain (INIB) and fetal liver/spleen (INFLS) libraries, from which a total of 45,192 and 86,088 expressed sequence tags, respectively, have been derived. While improving the representation of the longest cDNAs in our libraries, we developed three additional methods to normalize cDNA libraries and generated over 35 libraries, most of which have been contributed to our integrated Molecular Analysis of Genomes and Their Expression (IMAGE) Consortium and thus distributed widely and used for sequencing and mapping. In an attempt to facilitate the process of gene discovery further, we have also developed a subtractive hybridization approach designed specifically to eliminate (or reduce significantly the representation of) large pools of arrayed and (mostly) sequenced clones from normalized libraries yet to be (or just partly) surveyed. Here we present a detailed description and a comparative analysis of four methods that we developed and used to generate normalized cDNA libraries from human (15), mouse (3), rat (2), as well as the parasite *Schistosoma mansoni* (1). In addition, we describe the construction and preliminary characterization of a subtracted liver/spleen library (INFLS-SI) that resulted from the elimination (or reduction of representation) of -5000 INFLS-IMAGE clones from the INFLS library.

PMID: 889548 [PubMed - indexed for MEDLINE]

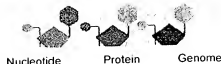
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☐ I: AA880300.vx39f05.r1 Strata...[gi:2989283]

Links

IDENTIFIERS

dbEST Id: 1607105
 EST name: vx39f05.r1
 GenBank Acc: AA880300
 GenBank gi: 2989283

CLONE INFO

Clone Id: IMAGE:1277601 (5')
 Source: IMAGE Consortium, LLNL
 DNA type: cDNA

PRIMERS

Sequencing: -28m13 rev1 ET from Amersham
 PolyA Tail: Unknown

SEQUENCE

CTTTCCTGGTAAACACCAAGTTTGAAGCGCTGACAGTTGTGATCCAGCAC
 GAGCGGGAGGAAGCACTGAAGCAACACAAAACCTCTCTCAAGAAGCTGTCT
 GGAGAGCTAGTTGCTGCTTCAAGCGCTGTGAGAAGCTAGAAAAAGGCTAGG
 CAGACAGCGTATCAAGAATTTGTCCAGAACTAAACAGCAGCATCAGACAC
 GAACTGGAGAACCAGCTGAAGGACTTATACACCGCAGAGTGTGAGAAGCTT
 TACATTGAGGAGGCAGAAAAATATAAACTCAACTGCAAGAGCAGTTTGAC
 GCCGCCCATGAGACCACTAAGCTTGAGATTGAAGCTAGCCACTCGGAGAAG
 CTGAAGAAGACCTATGAAACCTCCCTTTAGAAATCAAGAAGAGCCATGAG
 AAGTCACTGGAGGATCTGCT

Quality: High quality sequence stops at base: 464

Entry Created: Mar 26 1998
 Last Updated: Mar 26 1998

COMMENTS

This clone is available royalty-free through LLNL ;
 the IMAGE Consortium (info@image.llnl.gov) for further
 information.
 MGI:669401

LIBRARY

Lib Name: Stratagene mouse lung 937302
 Organism: Mus musculus
 Strain: C57BL/6 x CBA
 Sex: female
 Organ: lung
 Tissue type: lung
 Developmental stage: 6-8 month old

Vector: pBluescript SK-
R. Site 1: EcoRI
R. Site 2: XhoI
Description: Cloned unidirectionally. Primer: Oligo dT. 6-8 month
female lung and 1.5 year old male lung were source c
Average insert size: 1.5 kb; Uni-ZAP XR Vector; ~5'
sequence: 5' GAATTCGGCAGAG 3' ~3' adaptor sequence:
CTCGAGTTTTTTTTTTTTTTTTTTT 3'

SUBMITTER

Name: Marra M/Mouse EST Project
Lab: WashU-HHMI Mouse EST Project
Institution: Washington University School of MedicineP
Address: 4444 Forest Park Parkway, Box 8501, St. Louis, MO 63
Tel: 314 286 1800
Fax: 314 286 1810
E-mail: mouseest@watson.wustl.edu

CITATIONS

Title: The WashU-HHMI Mouse EST Project
Authors: Marra,M., Hillier,L., Allen,M., Bowles,M., Dietrich,
Dubuque,T., Geisel,S., Kucaba,T., Lacy,M., Le,M., Ma
Morris,M., Schellenberg,K., Steptoe,M., Tan,F., Unde
, Moore,B., Theising,B., Wylie,T., Lennon,G., Soares
Wilson,R., Waterston,R.
Year: 1996
Status: Unpublished

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LocusLink provides
curated information for
human, fruit fly, mouse,
rat, and zebrafish

Accession AA880300 was first seen at NCBI on Mar 27 1998
12:37 AM

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Entrez

BLAST

OMIM

Taxonomy

Structure

LAST 2 SEQUENCES RESULTS VERSION BLASTN 2.2.6 [Apr-09-2003]

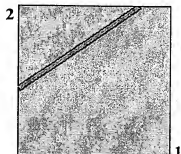
match: 1 Mismatch: 2 gap open: 5 gap extension: 2
 dropoff: 50 expect: 10.000 wordsize: 11 Filter ☐ Align ☐

sequence lc|seq_1

Length 354 (1 .. 354)

sequence gi vx39f05.r1 Stratagene mouse lung 937302 Mus musculus cDNA clone
 2989283 IMAGE:1277601 5'

Length 500 (1 .. 500)



OTE: The statistics (bitscore and expect value) is calculated based on the size of nr database

OTE: If protein translation is reversed, please repeat the search with reverse strand of the query sequence

Score = 535 bits (278), Expect = e-149
 Identities = 278/278 (100%)
 Strand = Plus / Plus

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Query: 1   catcagacagaccggacggaactggagaaccggctgaaggacttatacaccgcagagtgt 60
          |||
Sbjct: 223 catcagacagaccggacggaactggagaaccggctgaaggacttatacaccgcagagtgt 282

Query: 61   gagaagcttcagagcatttacattgaggaggcagaaaaatataaaactcaactgcaagag 120
          |||
Sbjct: 283 gagaagcttcagagcatttacattgaggaggcagaaaaatataaaactcaactgcaagag 342

Query: 121  cagtttgacaacttaaacgccgcccatgagaccactaagcttgagattgaagctagccac 180
          |||
Sbjct: 343 cagtttgacaacttaaacgccgcccatgagaccactaagcttgagattgaagctagccac 402

Query: 181  tcggagaaggtggaattgctgaagaagacctatgaaacctcccttcagaaatcaagaag 240
          |||
Sbjct: 403 tcggagaaggtggaattgctgaagaagacctatgaaacctcccttcagaaatcaagaag 462

Query: 241  agccatgagatggagaagaagtcactggaggatctgct 278
          |||
Sbjct: 463 agccatgagatggagaagaagtcactggaggatctgct 500
  
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PU time: 0.01 user secs. 0.01 sys. secs 0.02 total secs.

ambda	K	H
1.33	0.621	1.12

apped ambda	K	H
1.33	0.621	1.12

atrix: blastn matrix:1 -2
ap Penalties: Existence: 5, Extension: 2
umber of Hits to DB: 2
umber of Sequences: 0
umber of extensions: 2
umber of successful extensions: 1
umber of sequences better than 10.0: 1
umber of HSP's better than 10.0 without gapping: 1
umber of HSP's successfully gapped in prelim test: 0
umber of HSP's that attempted gapping in prelim test: 0
umber of HSP's gapped (non-prelim): 1
ength of query: 354
ength of database: 10,249,863,584
ffective HSP length: 24
ffective length of query: 330
ffective length of database: 10,249,863,560
ffective search space: 3382454974800
ffective search space used: 3382454974800
:
:
l: 6 (11.5 bits)
?: 26 (50.0 bits)
l: 12 (23.8 bits)
?: 20 (39.1 bits)

CURRICULUM VITAE
ARTHUR DONNY STROSBERG

PERSONAL INFORMATION:

Hybrigenics SA
3-5 Impasse Reille
75014 Paris (France)
Phone: 33 (0) 1 58 10 38 00
Fax: 33 (0) 1 58 10 38 40
Email: adstrosberg@hybrigenics.fr
Web site: <http://www.hybrigenics.com>

Address (home) 66, rue de Javel 75015 Paris (France)
Telephone: +33-1 45 79 52 42
Telefax: +33-1 45 77 76 98

Born: March 2, 1945 at Montreux (Switzerland)
Citizenship: Belgian
Marital Status: Married, two children

EDUCATION

1966 License in Chemistry
Free University of Brussels (Belgium)

1970 Doctorate in Sciences (Chemistry)
Free University of Brussels (Belgium)

PROFESSIONAL EXPERIENCE

1969-1972 Instructor in Protein Chemistry
Free University of Brussels (Belgium)

1970-1973 Research Fellow in Medicine
Massachusetts General Hospital, Boston (USA)

1972-1973 Instructor in Medicine, Harvard Medical School, Boston (USA)

1972-1977 Assistant Professor in Protein Chemistry
Free University of Brussels (Belgium)

1974-1980 Professor Biochemical Pathology
Free University of Brussels (Belgium)

1977-1990 Chair Professor Biochemistry and Immunology
University of Paris VII (France)

1982-1990	Chairman, Immunology Graduate School Ile de France
1982-1985	Chairman, Department of Biochemistry, University Paris VII (France)
1986-1990	Chief, Unit of Molecular Biology of Receptors (Institut Pasteur, Paris)
1986 -1990	Chairman of the Commission des Spécialistes, Section 37, University Paris VII, Conseil National des Universités.
1990 -1998	Director Unit of Molecular Immuno-Pharmacology, CNRS and Vice-President Institut Cochin de Génétique Moléculaire (Paris)
1993	Visiting Professor, Harvard Medical School (Boston, USA)
1998 -	Founder and Vice-President France Biotech (Communication and relation with research organisations)
1999 -	Founder, Chairman, President & CEO Hybrigenics

HONORS AND AWARDS:

1966-1968	I.R.S.I.A. - I.W.O.N.L. Fellowship
1970-1973	Fullbright Honorary Fellow
1970-1973 (EMBO)	Long-term Research Fellow European Molecular Organization
1974:	International Prize for Medical Research
1988	Knight in the Order of Merit
1992	Vermeil Medal of the Society for Encouragement of Progress
1992	Boehringer Ingelheim Lecturer
2001	Technology Pioneer World Economic Forum (Davos)

MEMBERSHIPS:

1969 -	Belgische Vereniging voor Biochemie (Belgie)
1972 -	New York Academy of Sciences
1973 -	American Association of Immunologists

1974 -	Nederlandse Vereniging voor Immunologie
1974 -	Société Française d'Immunologie
1975 -	The Biochemical Society
1976 - 1980	Inter-University Protein Sequence Group GISP-IGES (Founder and Chairman)
1978 - 1980	Belgian Society for Immunology (Founder and Chairman)
1979 - 1982	Associate Editor <i>Molecular Immunology (Immunochemistry)</i>
1983 -	European Molecular Biology Organization (EMBO)
1984 - 1989	Associate Editor <i>Molecular and Cellular Endocrinology</i>
1987 - 1995	Committee of French Drug Agency for approval of Biotechnology based drugs, Health Ministry
1988 - 1990	Associate Editor <i>Journal of Autoimmunity</i>
1990 -	Associate Editor <i>Current Opinion in Biotechnology</i>
1991 - 1994	Associate Editor <i>Vaccine Research</i>
1991 -	Associate Editor <i>Biologicals</i>
1991 - 1999	Associate Member of the Academy of Sciences Committee for the Applications of Sciences (C.A.D.A.S.)
1991 - 1994	Chairman, Scientific Committee of the Ministry of Research and Technology for Gene Therapy-based projects
1992 - 1994	European Editor of " <i>Receptors and Channels</i> "
1994 - 1997	Associate Editor <i>Immunological Investigations</i>
2003 -	Associate Editor <i>Clinical Proteomics</i>
2003 -	Associate Editor <i>Targets</i>
2003 -	Associate Editor <i>Genetic Engineering News</i>

COMMITTEE MEMBERSHIPS:

a. *Biotechnology Incubators (past and current)*

Paris BioParc (Business Incubator)

Genopole Entreprises

Paris Biotech (Cochin Institute)

BioTop (Pasteur Institute)

Biodiscovery (Rothschild group)

b. *General Interest*

CODEV (Committee for Paris Economic Development)

Government Fund of Funds (FPCR)

SITEF

MAJOR SCIENTIFIC ACHIEVEMENTS

- One of two laboratories to produce and sequence antibodies of restricted heterogeneity against microbial antigens pneumococcus, micrococcus (*Biochemistry*, 11, 4978-4985, 1972).
- First to produce monoclonal antibodies in 1973, by immortalizing, using SV40 rabbit lymphocytes, producing anti-pneumococcal antibodies (*Proc. Natl. Acad. Sci. USA* 71, 263-264, 1974).
- First to reveal the existence and to characterize "latent allotypes" in rabbits (*J. Immunol.* 113, 1313-1318, 1974; *Immunogenetics*, 4, 499-513, 1977).
- First to isolate, to separate from adenylyl cyclase and to characterize a G protein-coupled receptor: the β -adrenergic receptor from turkey erythrocytes (*Proc. Natl. Acad. Sci. USA* 74, 3710-3714, 1977).
- First to demonstrate that anti-idiotypic antibodies raised against anti-catecholamine antibodies, can recognize the β -adrenergic receptor (*J. Exp. Med.* 157, 1369-1378, 1983).
- One of two laboratories to first clone, sequence and express the human β -adrenergic receptor (*Proc. Natl. Acad. Sci. USA* 84, 6995-6999, 1987).
- First to achieve functional expression of G protein-coupled receptors in *E. coli* bacteria: β -adrenergic (*Proc. Natl. Acad. Sci. USA* 85, 7551-7555, [1988]; *EMBO J.* 9, 1471-1476, [1990]), 5HT_{1A} serotonin (*J. Biol. Chem.* 267, 8200-8206, 1992) and β -adrenergic receptor-G_s α fusion protein (*Proc. Natl. Acad. Sci. USA* 91, 8827-8831, 1994).
- First to clone, sequence and express the human β -adrenergic receptor (*Science* 245, 1118-1121, 1989), to describe its pharmacology (*Mol. Pharmacol.* 44, 1094-1104, 1993), its expression in human (*J. Clin. Invest.* 91, 344-349, 1993).
- One of two laboratories to first clone, sequence and express the human AT₂ angiotensin 1 receptor (*Eur. J. Biochem.* 220, 919-926, 1994) and to demonstrate coupling of its murine counterpart to a tyrosine phosphatase (*Receptors & Channels* 2, 271-280, 1994).
- One of the two laboratories to first identify a natural variant of human β -adrenergic receptor (*New Engl. J. Med.* 333, 343-347, 1995) and to correlate this single substitution with increased obesity (*New Engl. J. Med.* 333, 352-354, 1995).
- Peer-reviewed publications: 364
- Reviews and chapters in books: 75
- Books: 5

MAJOR INDUSTRY-RELATED ACHIEVEMENTS

- 21 issued patents in USA, Europe and other countries. Several licensed to major pharmaceutical companies.
- Major multiannual research and development contracts with international pharmaceutical companies including, Abbott, Bristol-Myers-Squibb, Ciba-Geigy, Hoffmann-LaRoche, Janssen Pharmaceutica, Servier. Sumitomo Chemicals.
- Multiannual personal consultancy agreements with, among others: Abbott, Genentech, Bristol-Myers-Squibb.
- Founder of several biotechnology companies including Chemunex SA (1984), Ideon/Incyte Inc. (1987/1993), Vetigen SARL (1992), Pharmaceutical Peptides Inc./Praecis (1994), Neurotech SA (1995), Hybrigenics (1998).
- Chairman, President and CEO Hybrigenics

Prof. A. Donny STROSBERG

publications 1969-2003

1. Strosberg, A.D. and Kanarek, L. "Immunochemical studies on hen's egg white lysozyme. Effect of formylation of the tryptophan residues." *FEBS Letters*, 5, 324-326 (1969).
2. Strosberg, A.D. and Kanarek, L. "Ratio of the Fab fragments I and II from goat antibodies and normal gamma globulins." *FEBS Letters*, 6, 28-30 (1970).
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5. Strosberg, A.D., Van Hoeck, B. and Kanarek, L. "Immunochemical studies on hen's egg-white lysozyme. Effect of selective nitration of the three tyrosine residues." *Eur. J. Biochem.*, 19, 36-41 (1971).
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RESEARCH

Normalization and Subtraction: Two Approaches to Facilitate Gene Discovery

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Large-scale sequencing of cDNAs randomly picked from libraries has proven to be a very powerful approach to discover (putatively) expressed sequences that, in turn, once mapped, may greatly expedite the process involved in the identification and cloning of human disease genes. However, the integrity of the data and the pace at which novel sequences can be identified depends to a great extent on the cDNA libraries that are used. Because altogether, in a typical cell, the mRNAs of the prevalent and intermediate frequency classes comprise as much as 50–65% of the total mRNA mass, but represent no more than 1000–2000 different mRNAs, redundant identification of mRNAs of these two frequency classes is destined to become overwhelming relatively early in any such random gene discovery programs, thus seriously compromising their cost-effectiveness. With the goal of facilitating such efforts, previously we developed a method to construct directionally cloned normalized cDNA libraries and applied it to generate infant brain (INIB) and fetal liver/spleen (INFLS) libraries, from which a total of 45,192 and 86,088 expressed sequence tags, respectively, have been derived. While improving the representation of the longest cDNAs in our libraries, we developed three additional methods to normalize cDNA libraries and generated over 35 libraries, most of which have been contributed to our Integrated Molecular Analysis of Genomes and Their Expression (IMAGE) Consortium and thus distributed widely and used for sequencing and mapping. In an attempt to facilitate the process of gene discovery further, we have also developed a subtractive hybridization approach designed specifically to eliminate (or reduce significantly the representation of) large pools of arrayed and (mostly) sequenced clones from normalized libraries yet to be (or just partly) surveyed. Here we present a detailed description and a comparative analysis of four methods that we developed and used to generate normalized cDNA libraries from human (15), mouse (3), rat (2), as well as the parasite *Schistosoma mansoni* (1). In addition, we describe the construction and preliminary characterization of a subtracted fetal liver/spleen library (INFLS-SI) that resulted from the elimination (or reduction of representation) of ~5000 INFLS-IMAGE clones from the INFLS library.

Large-scale single-pass sequencing of cDNA clones randomly picked from libraries has proven to be a powerful approach to discover genes (Adams et al. 1991, 1993a,b, 1995; Khan et al. 1992; McCombie et al. 1992; Okubo et al. 1992; Matubara and Okubo 1993; see also Hillier et al., this issue). However, the significance of using cDNA libraries that are well suited for this purpose should not be underestimated (Adams et al. 1993b).

Ordinary cDNA libraries may contain a high frequency of undesirable ("junk") clones (Adams et al. 1991, 1992) that may not only dra-

stically impair the overall efficiency of the approach, but also seriously compromise the integrity of the data that are generated. Among such junky clones are: (1) clones that consist exclusively of poly(A) tails of mRNAs; (2) clones that contain very short cDNA inserts; (3) clones that contain nothing but the 3' half of the NorI-oligo(dT)₁₈ primer used for synthesis of first-strand cDNA ligated to an adaptor; and (4) chimeric clones, i.e., cDNAs derived from different mRNAs joined artifactually during ligation. Furthermore, given that, as a general rule, the frequency of occurrence of a cDNA clone in a library is equivalent to that of its corresponding mRNA in the cell, even high-quality cDNA libraries may not be ideal for large-scale sequencing.

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Reassociation-kinetics analysis indicates that the mRNAs of a typical somatic cell are distributed in three frequency classes: (1) superprevalent (consisting of about 10–15 mRNAs that altogether represent 10–20% of the total mRNA mass); (2) intermediate (1000–2000 mRNAs; 40–45%); and (3) complex (15,000–20,000 mRNAs; 40–45%) (Bishop et al. 1974; Davidson and Britten 1979). Accordingly, once most mRNAs of the prevalent and intermediate frequency classes are identified, redundancy levels are expected to become greater than 60%. For this reason, the use of normalized libraries, in which the frequency of all clones is within a narrow range (Soares et al. 1994), has been shown to be beneficial for large-scale sequencing (Berry et al. 1995; Houlgate et al. 1995). Calculations show that at $C_0t = 5.5$ [where C_0 is the total DNA concentration and t is the time (moles nucleotides per liter \times sec)], of the three kinetic classes of mRNAs, the most abundant species are diminished drastically, while all frequencies are brought within the range of one order of magnitude (Soares et al. 1994).

However, because a large fraction of all human genes has been identified already, redundant identification of genes that are expressed in multiple tissues cannot be avoided simply by the use of normalized libraries. Hence, we argue that the use of subtractive cDNA libraries enriched for genes expressed at low levels and that have not yet been identified should become increasingly more advantageous for large-scale sequencing programs.

While attempting to improve the representation of the longest cDNAs in our libraries, we developed three methods for construction of normalized libraries, in addition to the procedure that we described previously (Soares et al. 1994), and used them successfully to generate normalized cDNA libraries from human (15), mouse (3), rat (2), and *Schistosoma mansoni* (1) tissues. All human and mouse cDNA libraries have been contributed to the Integrated Molecular Analysis of Genomes and Their Expression (IMAGE) Consortium (Lennon et al. 1996), and to date a total of 315,408 expressed sequence tags (ESTs) have been derived from these libraries (dbEST release 052396; <http://www.ncbi.nlm.nih.gov>).

Here we present a detailed description and a comparative analysis of the four methods that we have developed to normalize cDNA libraries; we describe a simple procedure for the construction of subtractive cDNA libraries; and we discuss

strategies that take advantage of subtractive hybridization to expedite the ongoing IMAGE/Washington University/Merck gene discovery program.

RESULTS

While attempting to improve the representation of the longest cDNAs in our normalized libraries, we developed four methods and constructed over 35 libraries, most of which are described here. A list comprising 15 human, three mouse, two rat, and one schistosome library with their respective names, number of recombinants, sequence tags, and methods used for normalization and preparation of single-stranded plasmids is shown in Table 1.

Extensive characterization of two normalized libraries [normalized infant brain (INIB) and normalized fetal spleen (INFLS)] constructed according to our previously described procedure (Soares et al. 1994; here designated as method 1) confirmed our original observations that a great extent of normalization can be achieved with this method for most cDNA species (e.g., cf. lanes 9,10 in Fig. 1M–P). It is noteworthy that the frequency of cDNA 122 (used as the probe in P) was increased with normalization from <0.0006% in the starting library to 0.007% in the INIB library (Soares et al. 1994). However, Southern hybridization of starting and normalized libraries with a battery of cDNA probes revealed that on occasion truncated clones were favored over their longest counterparts during the process. This was first observed when Southern blots of *NotI* + *HindIII*-digested plasmid DNA from starting and normalized infant brain libraries were hybridized with a cDNA probe for mitochondrial 16S rRNA (see Fig. 1L, lanes 9,10). Not only was the frequency of these mitochondrial cDNA clones not reduced effectively during the process of normalization (frequency of occurrence in starting and normalized infant brain libraries was 1.4% and 1.0%, respectively), but also the length of the hybridizing cDNAs was noticeably smaller in the normalized library: Comparative sequence analysis (not shown) of a number of hybridizing mitochondrial 16S rRNA clones from both starting and normalized libraries revealed that whereas the 3' end of most cDNAs derived from the starting library corresponded to the bona fide 3' end of the 16S rRNA, the 3' end of the majority of the cDNAs isolated from the normalized library corresponded to sequences further upstream on the

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Table 1. Complete List and Main Features of the Normalized Human, Mouse, Rat, and Schistosoma cDNA Libraries

mRNA source	Normalized library name	Number of recombinants in the normalized library	Preparation of single-stranded plasmids	Method of normalization	Library tag
Human infant brain ^a	1N1B	2,500,000	in vivo	1	AGCA
Human fetal liver spleen ^c	Nb2HFLS20W (1NFLS)	19,000,000	in vivo	1	ACATCT
	5Nb2HFLS20W	3,200,000	in vitro	2-1	
	6Nb2HFLS20W	4,400,000	in vitro	2-3	
	14Nb2HFLS20W	3,200,000	in vitro	4	
	15Nb2HFLS20W	35,000,000	in vitro	2-2	
Human term placenta	Nb2HP	750,000	in vitro	2-1	AGCA
Human 8-9W placenta	2NbHbpl-3NbHbpl	100,000	in vitro	2-3	GA
Human breast ^d	2NbHbpl-3NbHbpl	2,090,000	in vivo	2-1	CC
Human adult brain ^e	N2b4HBS5Y-N2b5HBS5Y ^g	3,170,000	in vivo	2-1	CC
Human retina ^h	2N2b4HR-N2b5HII	1,600,000	in vivo	2-1	AC
Human pineal gland ⁱ	3NbHPG	1,000,000	in vitro	2-1	CG
Human ovary tumor ^j	NbHOT	1,100,000	in vitro	2-1	GG
Human melanocyte ^k	2NbHM	8,800,000	in vitro	2-3	AG
Human fetal heart ^l	NbH119W	9,700,000	in vitro	4	ATC
Human parathyroid adenoma ^m	NbHPA	1,400,000	in vitro	4	ACCA
Human senescent fibroblast ⁿ	NbHSP	9,900,000	in vitro	4	ACCA
Human multiple sclerosis plaques ^o	2NbHMF	1,100,000	in vitro	3	CA
Human fetal lung ^p	NbH119W	21,700,000	in vitro	4	AA
19.5-dpc mouse embryo ^q	p3NMF19.5	3,400,000	in vitro	4	GACAC
17.5-dpc mouse embryo ^r	NbME17.5	6,800,000	in vitro	4	GGA
13.5- to 14.5-dpc mouse embryos ^s	NbME13.5-14.5	380,000	in vitro	4	GGA
Rat heart ^t	NbRH	400,000	in vitro	4	CAAC
Rat kidney ^u	2NbRK	130,000	in vitro	4	CAAC
8-week-old adult schistosoma ^v	NbSW	1,000,000	in vitro	4	GAAG

With the exception of 1N1B, which was constructed in the Lefmid BA vector, all libraries were constructed in the pT73-Pac vector. Cloning sites were NotI and EcoRI, except for fetal liver spleen (PstI and EcoRI) and infant brain (NotI and HindIII).

^aThe library tag is a sequence identifier present in the oligonucleotide used to prime the synthesis of first-strand cDNA, between the recognition sequence for the rare restriction enzyme (NotI or PstI) in the case of the liver spleen library used for directional cloning and the 3' end of the primer.

^bIn the human parathyroid adenoma, senescent fibroblast, mouse embryo, rat, and Schistosoma mansoni libraries located at the 3' end of the primer.

^cHuman infant brain (kindly provided by Dr. Conrad Gillen, Columbia University, New York, NY) was from a 72-day-old female who died in consequence of spinal muscular atrophy.

^dHuman fetal liver spleen (kindly provided by Dr. Stephen Brown, Columbia Presbyterian Medical Center, New York, NY) was from a 20-week-old postconception normal female.

^eTotal cellular poly(A)⁺ mRNA from normal breast pooled from reduction mammoplasty tissue was kindly provided by Dr. Anne Bowcock and Ms. Monique Spillman, University of Texas southwestern Medical Center at Dallas.

^f2NbHbpl differs from NbHbpl in the G₃ used for hybridization (23/ and 20, respectively).

^gTotal cellular adult brain RNA (kindly provided by Dr. Donald Glidier, University of Colorado Health Sciences Center, Denver) was obtained from a 55-year-old male who died of a ruptured aortic aneurysm. Brain tissue (frontal, parietal, temporal, and occipital cortex from the left and right hemispheres, subcalvarial white matter, basal ganglia, thalamus, cerebellum, midbrain, pons, and medulla) was acquired 17-18 hr after death.

^hNb2b4HBS5Y and Nb2b5HBS5Y differ from Nb2b4HBS5Y and Nb2b5HBS5Y, respectively, in the average size of their cDNA inserts (1.5-2.5 kb and 0.4-1.5 kb, respectively).

ⁱTotal cellular normal human retina RNA (kindly provided by Dr. Roderick R. McInnes, University of Toronto and Hospital for Sick Children, Canada) was obtained from a 55-year-old Caucasian male.

^jHuman pineal gland (kindly provided by Dr. David Klein, National Institute of Child Health and Human Development, National Institutes of Health (NIH)) was derived from a group of three pineal glands (gland 1: 18-year-old Caucasian male; gland 2: 18-year-old Caucasian female; gland 3: 29-year-old African American male).

^kTotal cellular human ovary tumor mRNA was kindly provided by Dr. Anne Bowcock and Ms. Monique Spillman, University of Texas southwestern Medical School. It was obtained from a 36-year-old Caucasian with a papillary serous cystadenocarcinoma grade III with surface extensions and metastases.

^lTotal cellular human melanocyte RNA (kindly provided by Dr. Anthony Albino and Dr. Alice de Oliveira, Memorial Sloan-Kettering Cancer Center, New York, NY) was derived from normal foreskin.

^mNormal human fetal heart and lung (kindly provided by Dr. Stephen Brown, Columbia Presbyterian Medical Center) were derived from the same 19-week-postconception specimen.

ⁿHuman parathyroid tumor (kindly provided by Dr. Stephen Marx, National Institute of Diabetes and Digestive and Kidney Diseases, NIH) was derived from sporadic adenomas.

^oCytoplasmic mRNA from senescent normal human fibroblasts was kindly provided by Dr. Barbara Suhart (National Institute of Environmental Health Sciences, NIH). The cells were prepared by pasturing normal human fibroblasts derived from foreskin until they exhibited an enlarged, flattened phenotype and failure to divide (labeling index of <2% following 48 hr-bromodeoxyuridine incorporation).

^pTotal cellular RNA from multiple sclerosis plaques (kindly provided by Dr. Kevin G. Becker, NINDS, NIH) was extracted from four lesions obtained from one patient.

^qTotal cellular RNA for construction of the mouse (C57BL/6J strain) embryonic libraries was kindly provided by Dr. Minori Ko (Wayne State University, Detroit, MI).

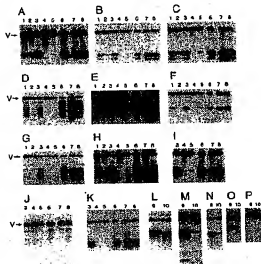
^rHeart tissues were obtained from an adult ZMC-Miller Sprague Dawley female and were kindly provided by Dr. Stephen Brown (Columbia Presbyterian Medical Center).

^sTotal cellular RNA from mature 8-week-old Schistosoma mansoni worms was kindly provided by Dr. Ron Blanton, Case Western Reserve University, Cleveland, OH.

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Figure 1 Comparative analysis of starting and normalized cDNA libraries by Southern hybridization with 14 cDNA probes. The 0.015 μ g *PacI* + *EcoRI* digested plasmid DNA from the starting fetal liver/spleen library (lane 6), from the normalized fetal liver/spleen libraries constructed according to method 2-1 (lane 7), method 2-3 (lane 2), method 2-2 (lane 3), method 1 (lane 4), method 4 (lane 5), and from the liver/spleen mini-libraries enriched for abundant cDNAs (HAP-bound fractions) generated with method 2-1 (lane 7) and method 4 (lane 8) were electrophoresed on 1% agarose gels, transferred to nylon membranes (GeneScreenPlus; DuPont/NEN) and hybridized at 42°C in 50% formamide, 5 \times Denhardt's solution, 0.75 M NaCl, 0.15 M Tris (pH 7.5), 0.1 M sodium phosphate, 0.1% sodium pyrophosphate, 2% SDS containing sheared and denatured salmon sperm DNA at 100 μ g/ml. Similarly, 0.05 μ g *NotI* + *HindIII* digested plasmid DNA from the starting (IB; lane 9) and normalized (1NIB; lane 10; method 1) infant brain libraries (Soares et al. 1994) were electrophoresed, transferred, and hybridized as described above. Radioactive probes were prepared by random primed synthesis using the Prime-It II kit (Stratagene). The following probes were used: α -globin (A), β -globin (B), γ -globin (C), serum albumin (D, shorter exposure; E, longer exposure), acidic ribosomal phosphoprotein PO (F), H19 RNA (G, shorter exposure; H, longer exposure), apolipoprotein A (I), angiotensinogen (J), protein PO (K), myelin basic protein (N), secretogranin (O), unknown cDNA 8 (K), mitochondrial 16S rRNA (L), α -Tubulin (M), myelin basic protein (N), secretogranin (O), and unknown cDNA 122 (P). All probes were contaminated intentionally with a small amount of vector DNA to enable visualization of vector bands and thus confirm that a similar amount of library DNA was loaded in all lanes. (V) vector band, which is released from the cDNA inserts by double digestion with the restriction enzymes specified above.



16S rRNA. The occurrence of such 3' truncations was also documented by sequence analysis (not shown) for serum albumin cDNAs in the fetal liver/spleen library (see Fig. 1D,E, lanes 4,6).

Reasoning that this problem could be circumvented if the fragments used in the hybridization with the single-stranded circles (1) were in excess, and (2) spanned the entire length of the cDNAs, we developed an alternative procedure to normalize cDNA libraries based on hybridization of *in vitro* synthesized RNA (driver) from an entire library with the library itself in the form of single-stranded circles (tracer) (see methods 2-1 and 2-2 in Fig. 2). Several normalized libraries were generated by this procedure (see Table 1).

Southern hybridization of endonuclease-restricted plasmid DNA from starting and normalized libraries with a number of cDNA probes (Fig. 1) indicated clearly that these methods effectively improved the representation of the longest cDNAs in the normalized libraries (e.g., cf. lanes 1,4 in Fig. 1A,D,E,G,H). However, characterization of one of these libraries (SNb2HFLS20W)

by colony hybridization with cDNA probes (not shown) indicated that this approach was effective to reduce the frequency of some, but not all, of the most abundant clones (e.g., serum albumin was reduced about 20-fold, whereas γ -globin was reduced only twofold). No difference was observed when hybridizations were performed at different conditions [0.4 M NaCl and 50% formamide at 42°C as in methods 2-1 and 2-3; 0.12 M NaCl, 50% formamide, and 1% sodium dodecyl sulfate (SDS) at 50°C as in method 2-2 (see lane 3 in Fig. 1); 0.4 M NaCl and 80% formamide at 42°C, not shown].

It is noteworthy that Northern hybridization (not shown) of *in vitro* transcribed RNA synthesized from an entire plasmid library with probes derived from the abundant cDNAs that failed to be normalized effectively by this procedure (e.g., globins in the fetal liver/spleen library and glyceraldehyde-3-phosphate dehydrogenase (G3PD) in the breast library) indicated that they were not as prevalent in the population of *in vitro* transcribed RNAs as they were in their respective starting cDNA libraries.

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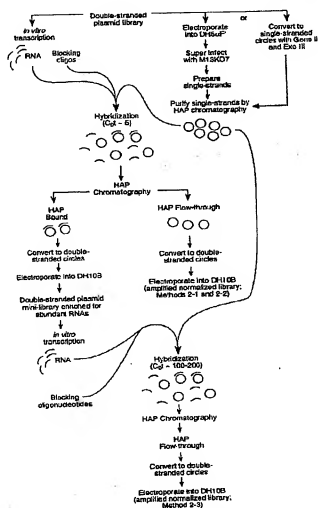


Figure 2 Diagram of the normalization methods 2-1, 2-2, and 2-3. Double-stranded plasmid DNA representing an entire starting library is (1) linearized with either *Sfi*, *Nci*, or *PacI* and used as template for synthesis of RNA in vitro using T3 or T7 RNA polymerases, and (2) converted to single-stranded circles either in vivo, upon electroporation into DH5 α and superinfection with M13KO7, or in vitro by the combined action of Gene II and Exonuclease III (Life Technologies). Single-stranded plasmid DNA is HAP-purified and hybridized ($C_{ot} = 5$) with excess RNA (pretreated with RNase-free DNase I; Promega), blocked with appropriate oligonucleotides to prevent hybridization through common vector sequences (see Methods section). Both the fraction that remains single-stranded (flow-through) as well as the resulting hybrids (bound) are purified by HAP chromatography. The HAP flow-through fraction is converted to double-stranded plasmids, electroporated into DH10B bacteria (Life Technologies), and propagated under ampicillin selection to generate an amplified normalized library (methods 2-1 and 2-2, depending on the conditions used for hybridization; see Methods section). The HAP-bound fraction is also converted similarly to double-stranded plasmids, electroporated into bacteria, and propagated under ampicillin selection to generate a mini-library enriched for abundant cDNAs. Double-stranded plasmid DNA from this mini-library is linearized and used as template for synthesis of RNA in vitro. After digestion of the plasmid DNA template with ribonuclease-free DNase I (Promega), the RNA (driver) is blocked with appropriate oligonucleotides and hybridized ($C_{ot} = 100-200$) with HAP-purified single-stranded plasmids derived from the starting library (see above). The remaining

single-stranded circles are purified by HAP chromatography, converted to double-stranded circles, electroporated into DH10B (Life Technologies), and propagated under ampicillin selection to generate an amplified normalized library (method 2-3).

A significantly improved extent of normalization was achieved when runoff RNA synthesized from the plasmid mini-library enriched for abundant cDNAs (hydroxyapatite (HAP)-bound fraction of method 2-1 in Fig. 2) was hybridized ($C_{ot} = 100-200$) with single-stranded circles from the starting library (see method 2-3 in Fig. 2 and Table 1; cf. lanes 1,2 in Fig. 1A-D,F,G).

In an effort to preserve the positive charac-

teristics of both methods 1 and 2 (i.e., the adequate extent of normalization achieved with method 1, and the improved representation of the longest cDNAs achieved with method 2), we developed two additional reassociation kinetics based procedures involving DNA-DNA hybridization (methods 3 and 4; see Fig. 3).

Method 3, which was successfully used to construct a normalized library from multiple

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sclerosis plaques (see 2NbHMSP in Table 1). Involved hybridization of a 20-fold excess of single-stranded cDNA fragments (comprising the 5' halves of all inserts of the starting library, generated by Exonuclease III digestion of gel-purified double-stranded cDNAs; see Fig. 3) with complementary single-stranded circles produced in vitro by the combined action of Gene II and Exonuclease III (Life Technologies).

Southern hybridization of *NotI* + *EcoRI*-digested plasmid DNA from the starting and normalized (with methods 2-1 and 3) multiple sclerosis plaques library with mitochondrial 16S rRNA and myelin basic protein cDNA probes (not shown) clearly indicated that method 3 was superior to method 2-1 in that a much greater extent of normalization was achieved, at the same time that it maintained (similar to method 2-1) appropriate representation of the longest cDNAs in both cases.

For the libraries constructed with method 4 (see Table 1 and Fig. 3), double-stranded cDNA inserts generated by the polymerase chain reaction (PCR) with T3 and T7 primers were melted and hybridized (in the presence of vast excess of blocking oligonucleotides) with single-stranded plasmid library DNA prepared in vitro.

Southern hybridization of *PacI* + *EcoRI*-digested plasmid DNA from starting and normalized (with methods 1, 2-1-2-3, and 4) fetal liver/spleen libraries (Fig. 1) with several cDNA probes (including those that revealed incomplete normalization with methods 2-1-2-3, such as α -globin, β -globin and γ -globin) demonstrated the efficacy of method 4 in achieving the desired extent of normalization obtained with method 1 (cf. lanes 1-6 in Fig. 1A-D, F-H, and lanes 3-6 in Fig. 1I-K) while preserving the representation of the longest cDNAs (e.g., the longest albumin cDNA was present in the normalized library prepared with method 4, shown in lane 5 of Fig. 1D,E, but it was undetectable in the normalized library constructed with method 1, shown in lane 4; a similarly remarkable difference was revealed with the cDNA probe for H19 RNA, shown in Fig. 1G,H). Characterization of the normalized library generated with method 4 by colony hybridization with 10 cDNA probes (not shown), which occur at a wide range of frequencies in the starting library, confirmed the effectiveness of the procedure to narrow their frequencies down to within one order of magnitude (e.g., the frequencies of the cDNAs for γ -globin, α -globin, β -globin, H19 RNA, and transferrin were reduced

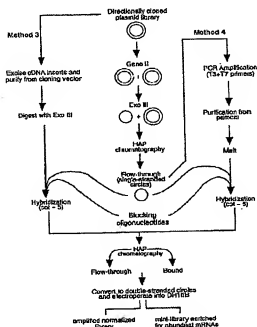


Figure 3 Diagram of the normalization methods 3 and 4. In method 3 double-stranded plasmid DNA from a starting library is digested with restriction enzymes that generate 5' protruding ends, and the excised cDNA inserts are gel-purified from the cloning vector and digested with Exonuclease III to yield noncomplementary single-stranded fragments, each representing half of a cDNA insert. Note that the single-stranded fragments that span the 5' half (but not the 3' half) of the cDNA inserts are complementary to single-stranded plasmids prepared in vitro. These single-stranded DNA fragments are blocked with appropriate oligonucleotides (see Methods) and hybridized with single-stranded library DNA prepared in vitro (middle column). The remaining single-stranded circles are HAP-purified, converted to double-stranded plasmids, electroporated into DH10B bacteria (Life Technologies), and propagated under ampicillin selection to generate a normalized library. In method 4, single-stranded library DNA is used as template for PCR amplification with T3 and T7 primers. PCR-amplified cDNAs are purified from excess primers, melted, and hybridized with single-stranded library DNA in the presence of blocking oligonucleotides. The remaining single-stranded circles are purified by HAP chromatography, converted to double-stranded plasmids, electroporated into bacteria, and propagated under ampicillin selection to generate a normalized library.

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from 9.2%, 6.4%, 3.6%, 1.8%, and <0.2% to 0.04%, 0.02%, 0.01%, 0.1% and 0.1%, respectively).

In order to assess further the ability of these normalization procedures to preferentially reduce the representation of the most abundant cDNAs, we have performed a comparative sequence analysis (not shown) of 100 clones picked randomly from the fetal liver/spleen cDNA library normalized with method 4 (14Nb2HFLS20W in Table 1; HAP-flow-through fraction in Fig. 3), and from two fetal liver/spleen mini-libraries enriched for abundant cDNAs (HAP-bound fractions in Figs. 2 and 3) obtained during HAP purification of the normalized libraries prepared according to methods 2-1 (5Nb2HFLS20W) and 4 (14Nb2HFLS20W). A number of cDNAs known to be prevalent in the starting fetal liver/spleen library (e.g., albumin, γ -globin, α -globin, β -globin, mitochondrial RNAs, and apolipoproteins A and H) were found at increased frequencies in both mini-libraries enriched for abundant cDNAs, but none of them was represented in the sample of 100 clones from the normalized library. It is noteworthy that while 47% of the sequences derived from the normalized library were not represented in the "all nonredundant" subdivision of sequences of GenBank + EMBL + DDBJ + PDB, the majority of the sequences obtained from the mini-libraries of abundant cDNAs derived from methods 2-1 and 4 (91.4% and 86.9%, respectively) did have homologous sequences in that data base. Furthermore, although 49% of the sequences derived from the normalized library had fewer than 10 homologous ESTs in the dbEST subdivision of GenBank, most of the sequences obtained from both mini-libraries had greater than 10 homologous ESTs in the dbEST data base (92.5% and 89.7%, respectively, in the HAP-bound fractions of methods 2-1 and 4).

With the ultimate goal of facilitating the ongoing process of gene discovery by large-scale sequencing of cDNA clones picked randomly from libraries, we have performed a pilot subtractive hybridization experiment to eliminate (or reduce representation of) a pool of approximately 5000 IMAGE Consortium-arrayed cDNA clones (pool no. 1, LLAM 78-90) from the normalized library from which they were derived (1NFLS in Table 1). PCR-amplified cDNA inserts from pool no. 1 were melted and hybridized, in the presence of blocking oligonucleotides, with single-stranded plasmid DNA from the 1NFLS library, prepared *in vitro*. The remaining single-stranded circles were purified by HAP chromatography, converted to

double-stranded plasmids, electroporated into bacteria, and propagated under antibiotic selection to generate the subtracted 1NFLS-S1 library (see Fig. 4). Preliminary characterization of the 1NFLS-S1 library by Southern hybridization with 10 cDNA probes (only five are shown; see Fig. 5) known to be represented in pool no. 1 indicated clearly the effectiveness of the procedure to eliminate (or to reduce the representation of) all 11 cDNA sequences in the 1NFLS library. A BLASTN search of the dbEST division of GenBank (6/12/96) with 3' ESTs obtained from the five probes (cDNAs -1, -4, -8, -9, and -10) the hybridizations of which were not shown in Figure 5, revealed the presence of 0, 0, 1, 2, and 2 corresponding ESTs, respectively, from the 1NFLS library, thus indicating that the subtraction was successful even for cDNAs that were under-represented in the normalized library (a total of 44,407 3' ESTs have been derived from the 1NFLS library to date). It should be noted that because of sequencing failures, some of the clones in these arrays may not yet have corresponding ESTs in the public data bases.

It is noteworthy that when we attempted to perform the same subtractive hybridization experiment using, as driver, RNA synthesized *in vitro* from a plasmid DNA preparation of pool no. 1, the results obtained were not satisfactory (not shown) in that subtraction could be demonstrated for some but not all tested clones (e.g., α -globin could not be subtracted effectively), similar to what we observed in normalizations with method 2-1.

DISCUSSION

As a result of an effort to improve the representation of the longest cDNAs in our normalized libraries, we have developed four different methods for normalization of directionally cloned cDNA libraries constructed in phagemid vectors, while contributing resources to the IMAGE Consortium (Lennon et al. 1996) and thereby facilitating the ongoing gene discovery and mapping programs. Approximately 87.5% of all (human) IMAGE ESTs were derived from the normalized libraries described here.

The normalization procedure (method 1) that we described previously (Soates et al. 1994) was applied for the construction of the 1NIB and 1NFLS normalized libraries, from which a total of 45,192 and 86,088 ESTs, respectively, have been derived (dbEST release 052396; <http://>

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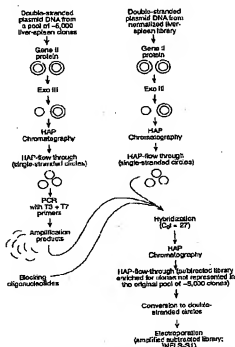


Figure 4 Diagram of the subtractive hybridization procedure used to generate the 1NFLS-S1 library. Double-stranded plasmid DNA from a pool of ~5000 IMAC Consortium-arrayed cDNA clones derived from the 1NFLS library (pool no. 1, LLAM 78-90) was converted to single-stranded circles in vitro by the combined action of Gene II and Exonuclease III (Life Technologies). The resulting single-stranded plasmids were HAP-purified and used as a template for PCR amplification with T3 and T7 primers. PCR-amplified cDNA inserts were purified from excess primers, melted, and hybridized with single-stranded circles (prepared in vitro) from the 1NFLS library, in the presence of appropriate blocking oligonucleotides. The remaining single-stranded circles were purified by HAP chromatography, converted to double-stranded plasmids, electroporated into DH10B bacteria (Life Technologies), and propagated under ampicillin selection to generate the (1NFLS-S1) subtracted library.

www.ncbi.nlm.nih.gov). Data analysis (see Hillier et al., this issue) demonstrated solidly the efficacy of this approach in bringing the frequency of all clones to within a narrow range. Extensive characterization of these two libraries by Southern analysis, however, revealed that on

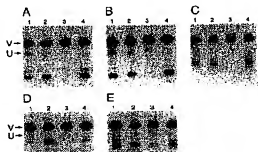


Figure 5 Characterization of the 1NFLS-S1 subtracted liver/spleen library by Southern hybridization with 5 cDNA probes. The 0.15 µg *PacI* + *EcoRI*-digested plasmid DNA from the fetal liver/spleen library normalized with method 1 (1NFLS; lane 1), from the pool of ~5000 IMAC Consortium-arrayed cDNA clones derived from the 1NFLS library (pool no. 1, LLAM 78-90; lane 2), from the subtracted library generated according to the diagram shown in Fig. 4 (1NFLS-S1; lane 3), and from the HAP-bound fraction obtained during HAP purification of the 1NFLS-S1 library (see Fig. 4) were electrophoresed, transferred to nylon membranes, and hybridized as described in the legend to Fig. 1. The following cDNA probes were used: α-globin (A), γ-globin (B), serum albumin (C), unknown cDNA 7 (D), picked randomly from pool no. 1, LLAM 78-90, and unknown cDNA 5 (E, picked randomly from pool no. 1, LLAM 78-90). A BLASTN search of the dbEST subdivision of Genbank with 3' ESTs derived from cDNA 7 and cDNA 5 revealed the presence of 33 and 0 corresponding ESTs, respectively, from the 1NFLS library. All probes were contaminated intentionally with a small amount of vector DNA to enable visualization of vector bands and thus confirm that a similar amount of library DNA was loaded in all lanes. (V) vector band; (U) residual undigested plasmid.

occasion truncated clones were favored over their longest counterparts during the normalization procedure.

Because of the relatively permissive conditions used for synthesis of first-strand cDNA, priming with the *NotI*-tag-(dT)₁₈ oligonucleotide may occur not only at the poly(A) tail of the mRNAs but also at internal A-rich sites within the mRNAs (e.g., at Alu tails). Typically, cDNAs with 3' truncations occur at frequencies of 10–15% in directionally cloned libraries. Truncated clones can be recognized (tentatively) as such, by the absence of a bona fide polyadenylation signal

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sequence at the appropriate distance upstream from the oligo(dA)₁₈ tail of the cDNA.

Why may truncated cDNAs be favored over their longest counterparts during normalization by method 1? Briefly, method 1 (Soares et al. 1994) involves: (1) annealing of a single-stranded DNA preparation of a directionally cloned cDNA library with an oligo(dT)₁₈ primer; (2) controlled primer extension reactions in the presence of deoxynucleotides and dideoxynucleotides to generate 3' noncoding extension products of approximately 200–300 nucleotides; (3) purification of the resulting partially double-stranded circles by HAP chromatography; (4) melting and reassociation of the HAP-purified partially double-stranded circles to a relatively low *C*₀t (5–10); (5) purification of the remaining single-stranded circles (normalized library) over HAP; (6) conversion of the single-stranded circles to double-stranded circles; and (7) electroporation into bacteria.

It could be anticipated that during the reassociation reaction, because truncated cDNAs occur at lower frequencies than their nontruncated counterparts, the extension products of the truncated cDNAs would more likely reanneal to the nontruncated overlapping cDNAs than to their own truncated templates. On the other hand, the extension products of the nontruncated cDNAs would most likely reassociate to their own nontruncated templates not only because they are more prevalent but also because of the low probability of there being an overlap between the short extension product of a nontruncated clone and a truncated single-stranded circle. As a result, nontruncated single-stranded circles are more likely to end up reassociated with more than one (nonoverlapping) extension product, whereas their truncated counterparts would remain single-stranded and therefore end up in the HAP flow-through fraction (normalized library).

Reasoning that this problem could be circumvented if the hybridizing fragments (1) were in excess over single-stranded circles, and (2) spanned the entire length of the cDNAs to maximize the opportunity of overlap between truncated and nontruncated clones, we devised an approach (methods 2-1 and 2-2; note that 2-2 is the same as 2-1 except that hybridization conditions were different) whereby *in vitro* synthesized RNA from a plasmid DNA preparation of a starting library is used as driver in hybridization (*C*₀t = 5) with the same library in the form of single-stranded circles. Indeed, these modifica-

tions improved successfully the representation of the longest cDNAs in the normalized libraries (e.g., serum albumin in the liver/spleen libraries).

However, in every library constructed with methods 2-1 and 2-2, we were able to identify cDNA clones that seemed to become normalized with much greater difficulty than others (e.g., α -globin in the 5Nb2HLS20W liver/spleen library, and G3PD in the breast library). We interpreted these results as suggestive that not all clones might be transcribed *in vitro* with the same efficiency if in a mixture (i.e., *in vitro* transcription of plasmid DNA from an entire library), and/or secondary structures in the RNAs (or interactions between RNAs) might impair their ability to hybridize with the single-stranded circles. These hypotheses were corroborated by the observation (not shown) that relatively weak hybridization signals were observed when Northern blots of RNA transcribed *in vitro* from an entire plasmid library were hybridized with cDNA probes derived from those clones that could not be normalized as effectively, despite the fact that they occurred at high frequencies in the starting libraries from which the *in vitro* transcribed RNAs were synthesized. We did exclude the possibility that the clones that were not being normalized effectively carried deletions that prevented them from being transcribed appropriately *in vitro* (not shown). In fact, all clones that were tested individually for *in vitro* transcription yielded the expected amounts of full-length RNA. Although this problem was significantly minimized in method 2-3 (cf. lanes 1,2 in Fig. 1A-D,F,G) the extent of normalization that was achieved was still not comparable to that obtained with method 1 (cf. lanes 2,4 in Fig. 1A-D,F,H).

The advantage of method 2-3 over methods 2-1 and 2-2 is that the RNA driver is derived from a mini-library (of relatively low complexity) enriched for abundant cDNAs rather than from the entire starting library. For this reason, higher *C*₀t hybridizations can be carried out to eliminate or reduce significantly the representation of the most abundant cDNAs. It should be noted, however, that method 2-3 is not a true normalization procedure, because the aim of this approach is not to equalize the frequency of all cDNA clones but rather to reduce significantly (or even to eliminate, depending on the *C*₀t used) the representation of the most abundant clones.

The extent to which the enrichment for abundant transcripts can be achieved in such

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mini-libraries depends essentially on the C_{0t} used for reassociation. Calculations based on estimates of frequencies of brain mRNAs (Soares et al. 1994) indicate that the best enrichments are obtained at a C_{0t} = 5–10. If the C_{0t} is too low (≤ 1) the enrichment is only for the most prevalent (class I) mRNAs; there is no enrichment for the mRNAs of the intermediate frequency class (class II) mRNAs. On the other hand, if the C_{0t} is too high (≥ 50) the enrichment for class I transcripts starts to become less significant because of a higher representation of mRNAs of the complex class (class III). Prevalent and Intermediate (classes I + II) brain mRNAs comprise 93–95% of the total cDNA population in a C_{0t} = 5–10 HAP-bound mini-library, in contrast to 62% in the starting library. Consequently, the frequency of class III transcripts in a C_{0t} = 5–10 HAP-bound mini-library is about 5.5-fold lower than that of the starting library (5–7% in the bound mini-library vs. 38% in the starting library).

Methods 3 and 4 were developed as a result of an attempt to achieve both the adequate extent of normalization obtained with method 1 and the improved representation of the longest cDNAs accomplished with methods 2-1, 2-2, and 2-3. Although more technically cumbersome, method 3 is superior to method 4 in that the DNA driver used in the hybridization is single-stranded.

Single-stranded driver in method 3 (see Fig. 3) is generated by Exonuclease III digestion of gel-purified double-stranded cDNA inserts excised from the starting library. The resulting non-complementary single-stranded fragments represent the 5' and 3' halves of the original cDNA inserts. The fragments that correspond to the 5' halves of the cDNAs are complementary to single-stranded circles prepared *in vitro*, whereas the single-stranded fragments that correspond to the 3' halves of the cDNA inserts are complementary to single-stranded plasmids prepared *in vivo*. Note that for the multiple sclerosis plaques library constructed with method 3 we used single-stranded circles prepared *in vitro*.

Production of single-stranded circles *in vitro* by the combined action of Gene II and Exonuclease III (Life Technologies), rather than *in vivo* by superinfection of a culture with a helper phage, is very beneficial because it circumvents the distortions that otherwise may arise as a result of the differential growth properties of clones with different size inserts. However, because the digestion with Gene II results in the conversion of

most, but not all, supercoiled plasmids to relaxed circles, it becomes necessary to purify the single-stranded circles that are produced after digestion with Exonuclease III by HAP chromatography.

For construction of the normalized multiple sclerosis plaques library, the cDNA inserts were excised by double digestion of plasmid DNA from the starting library with *NorI* and *EcoRI*. The fact that one in every three clones might have an internal *EcoRI* site (an *EcoRI* site is expected to occur once every 4096 bp, and the average insert size in these libraries is of the order of 1.4 kb) should not compromise the efficiency of the procedure, because at least one of the resulting restriction fragments would be expected to be ≥ 200 bp (clones smaller than 400 bp are size-selected out of these libraries) and therefore be able to form hybrids that would bind quantitatively to HAP under our conditions. A disadvantage to method 3, as presented, is that only clones < 2.9 kb (approximate vector size) can be excised cleanly from the vector. It is conceivable, however, that one might be able to use double-stranded cDNA fragments generated by PCR amplification with T3 and T7 primers as substrate for the Exonuclease III digestion in method 3.

Method 4 was used to generate a significant fraction of the libraries that were contributed to the IMAGE Consortium (see Table 1). It is undoubtedly the simplest and overall most advantageous of all procedures. Because the DNA driver is generated by PCR amplification of the starting (double-stranded or single-stranded, see below) plasmid library with T3 and T7 primers, the tracer (single-stranded circles) used in this hybridization may be produced *in vitro* or *in vivo*.

The extent of normalization achieved with method 4 was comparable to that obtained with method 1 with the advantage that it successfully preserved the representation of the longest cDNAs (cf. lanes 4,5 in Fig. 1). Moreover, method 4 is superior to method 1 because it does not preclude the clones derived from mRNAs with internal *NorI* sites from being represented in the normalized library. Because the starting material for the reassociation kinetics reaction in method 1 is generated by a controlled primer extension reaction with an oligo(dT)₁₈ primer, clones without an oligo(dA)₁₈ tail (derived from mRNAs with an internal *NorI* site) are not represented in the final normalized library, although they are not necessarily lost (clones without tails end up in the HAP flow-through fraction during HAP purification of the partially double-stranded circles

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generated by this primer extension reaction). It should also be noted that this problem of method 1 could be circumvented by the use of an oligonucleotide complementary to flanking vector sequences (as opposed to the oligo(dT)₁₈ for this controlled primer extension reaction).

The potential biases introduced by PCR amplification in method 4 are minimized by the fact that (1) PCR amplification products are used in excess in these hybridizations, and (2) the size distribution of inserts in these libraries is relatively narrow (ranging typically from 0.4 to 2.5 kb).

The conditions used for hybridization greatly influenced the quality of the resulting normalized libraries constructed with method 4. This is to a great extent a consequence of the fact that we are using HAP to purify single-stranded circles, as opposed to a biotin-avidin capture system, which in our hands yielded significantly less satisfactory results (M.F. Bonaldi and M.R. Soares, unpubl.). The best results were obtained when the hybridization conditions were the most similar to the HAP conditions. We interpreted these results as suggestive of the fact that imperfect hybrids formed during hybridization may either not bind to HAP and/or may melt once in the HAP buffer.

It is noteworthy that a much superior extent of normalization was obtained with method 4 when single-stranded plasmid DNA prepared in vitro, as opposed to double-stranded plasmid DNA, was used as template for PCR amplification (not shown). These results suggest that a fraction of the double-stranded plasmids used as template for PCR amplification, presumably in the form of melted supercoiled DNA, might end up in the HAP flow-through fraction (normalized library) during purification.

It is noteworthy that cross-hybridizing diverged sequences seem to escape normalization in all of the procedures discussed above. For example, the frequency of Alu repeat-containing cDNAs (typically 10% in directionally cloned cDNA libraries) is practically the same in starting and normalized libraries. These results suggest that imperfect hybrids either do not bind to HAP under our conditions or melt once diluted in the (more stringent) HAP buffer. This is advantageous, not only because it preserves the representation of Alu-containing cDNAs that might correspond to otherwise rare mRNAs, but also, and most significant, because it minimizes the likelihood that a rare member of a gene family might be excluded from the final (normalized or subtracted) library as a result of a cross-hy-

bridization with a more prevalent but diverged sequence.

The use of normalized libraries for large-scale gene discovery/EST programs is beneficial because it minimizes redundancies while increasing the representation of the rarer cDNAs by about threefold, on average. However, given the great extent of overlap in gene expression among different tissues, the use of normalized libraries alone is not sufficient to maintain a desirable pace of identification of novel sequences at advanced stages of such programs. For this reason, we propose that the use of subtracted libraries enriched for clones not yet identified might become increasingly advantageous. Toward this goal, we have developed a subtractive hybridization approach designed specifically for this purpose (see Fig. 4). In a pilot experiment, we were able to reduce significantly the representation of ~5000 INFLS-IMAGE Consortium clones from the INFLS library itself (see Fig. 5). With the development of appropriate clustering algorithms, the use of nonredundant sets of cDNA/gene sequences as drivers for hybridizations to generate subtractive libraries enriched for novel sequences should soon become possible, and hopefully will facilitate the isolation of all human and mouse cDNAs still awaiting identification.

METHODS

Construction of Directionally Cloned cDNA Libraries

Poly(A)⁺ RNA was purified from total cellular RNA (except for senescent fibroblasts from which cytoplasmic RNA was isolated) using the Oligotex mRNA kit (Qiagen) according to the manufacturer's instructions, except that two rounds of purification were performed. cDNA library construction was essentially as described before (Adams et al. 1993b; Soares 1991). Typically, 1 µg poly(A)⁺ RNA was annealed at 37°C with a twofold molar excess of a NotI-tag-(dT)₁₈ primer [or EcoI-tag-(dT)₁₈ in the case of the liver/spleen library] and reverse transcribed at 37°C with Superscript Reverse Transcriptase (Life Technologies). Alternatively poly(A)⁺ RNA was annealed at 45°C with a fourfold molar excess of a NotI-tag-(dT)₂₄ primer and reverse transcribed at 45°C. The tag is a sequence of 2–6 nucleotides that is unique for each library and thus serves as an identifier (see Table 1). With the exception of infant brain, fetal liver/spleen and term placenta, all other first-strand cDNA syntheses were primed with the following oligonucleotide: TGTTACGAATCTGAAGTGGAGCGCGCG-tag-(dT)₁₈ or ₂₄. The oligonucleotide AACTGGAAGAATTCGGCGCGGAGGAA(dT)₁₈ (Pharmacia) was used to prime both infant brain and term placenta first-strand cDNA syntheses. The oligonucleotide AACTGGAAGAATTAATAA-GATCT(dT)₁₈ was used to prime the synthesis of first-

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strand fetal liver/spleen cDNA. Double-stranded cDNAs were size-selected by gel filtration over a long (64-cm) and narrow (0.2-cm diameter) Bio-Gel A-50m (Bio Rad, 100-200 mesh) column, and ligated to a 500- to 1000-fold molar excess of adapters. Infant brain cDNAs were ligated to *Hind*III adapters, digested with *NorI*, size selected over a second Bio-Gel column, and cloned directionally into the *NorI* and *Hind*III sites of the pLacZ vector (Sears et al. 1994). Fetal liver/spleen cDNAs were ligated to *Eco*RI adapters (Pharmacia), size-selected as above, digested with *PacI* and cloned directionally into the *PacI* and *Eco*RI sites of the pT773-*Pac* vector. All other cDNAs were ligated to *Eco*RI adapters (Pharmacia), size-selected as above, digested with *NorI* and cloned directionally into the *NorI* and *Eco*RI sites of the pT773-*Pac* vector. pT773-*Pac* is essentially the same as pT77318D (Pharmacia) with a modified polylinker. Figure 6 shows the sequence of the pT773-*Pac* polylinker and flanking sequences.

Production of Purified Covalently Closed Single-stranded Library DNA In Vitro

Double-stranded phagemid DNA was converted to single-stranded circles by the combined action of Gene II (phage ϕ 1 endonuclease) and *Escherichia coli* Exonuclease III enzymes, as per the manufacturer's instructions (Life Technologies; cat. no. 10356-020). The resulting single-stranded circular DNA was purified from the remaining double-stranded plasmids by HAP chromatography (Bio-Rad) as described previously (Sears et al. 1994). The replication initiator protein of bacteriophage ϕ 1 (Gene II) is a site-specific endonuclease that binds to the ϕ 1 origin in phagemid vectors and nicks the viral strand of the supercoiled DNA. The nicked strand is then digested from its 3' end with Exonuclease III (Hohelsel 1993) to generate single-stranded circles. Purification of the resulting single-stranded circles over HAP is necessary because the conversion of supercoiled to relaxed plasmids by Gene II is never complete. The Gene II reaction was performed for 1 hr at 30°C and contained typically 4 μ g supercoiled plasmid library DNA, 1 μ l Gene II (Life Technologies), and 2 μ l 10 \times Gene II buffer (Life Technologies) in a total volume of 20 μ l. The Gene II protein was heat inactivated for 5 min at 65°C; the reaction mixture was chilled on ice; 2 μ l Exonuclease III (Life Technologies, Cat. No. 18013-011, 65 units/ μ l) was added; and the reaction was incubated for 30 min at 37°C. Gene II and Exonuclease III were then digested with Proteinase K (Boehringer Mannheim) for 15 min at 50°C in a 100- μ l reaction containing 10 mM Tris

(pH 7.8), 5 mM ethylenediamine tetraacetic acid (EDTA), 0.5% SDS, and 136 μ g Proteinase K. After extraction with equal volume of phenol-chloroform-isoamyl alcohol (25:24:1), library DNA was ethanol-precipitated and digested with *PvuII* for 2 hr at 37°C. This was done to convert the remaining supercoiled plasmids into linear DNA molecules and thereby improve their bindability to IAP under our conditions. Note that *PvuII* does not cleave single-stranded circles and that there are two *PvuII* sites in the vector. The reaction was diluted with 2 ml loading buffer [0.12 M sodium phosphate buffer (pH 6.8), 10 mM EDTA, and 1% SDS] and purified by HAP chromatography at 60°C, using a column pre-equilibrated with the same buffer (1 ml bed vol; 0.4 g of HAP). After a 6-ml wash with loading buffer, this volume was combined with the flow-through fraction, and the sample was extracted twice with water-saturated 2-butanol, once with dry 2-butanol, and once with water-saturated ether (3 vols. per extraction). Residual ether was blown off by vacuum and the sample was desalted by passage through a Nucleo column (DuPont/NEN) according to the manufacturer's specifications, concentrated down to ~0.35 ml and ethanol-precipitated. Note that Gene II-Exonuclease III prepared single-stranded DNA is in the opposite polarity to single stranded DNA generated by in vivo phagemid production.

Production of Purified Covalently Closed Single-stranded Library DNA In Vivo

Plasmid DNA from the starting library was electroporated into *E. coli* DH5 α bacteria, and the culture was grown under ampicillin selection at 37°C to an OD₆₀₀ of 0.2, supplemented with a 10- to 20-fold excess of the helper phage M13KO7 (Pharmacia), and harvested after 4 hr for preparation of single-stranded plasmids, as described (Vieira and Messing 1987).

Conversion of Single-stranded Circles to Double-stranded Plasmids

Single-stranded circles (<50 ng) were ethanol-precipitated and resuspended in 11 μ l water. Then 4 μ l 5 \times Sequenase buffer (USB) and 1 μ l primer (1 μ g) were added and the mixture was incubated at 65°C for 3 min and then at 37°C for 3 min. Then 1 μ l Sequenase version 2.0 (USB), 1 μ l 0.1 M dithiothreitol (DTT), and 2 μ l mixed dNTP stock (a solution containing each deoxynucleotide at a final concentration of 10 mM) were added, and the reaction was incubated at 37°C for 30 min. The total volume was taken up to 100 μ l with 10 mM Tris (pH 8.0) and 1 mM EDTA (TE) and the reaction was extracted once with phenol-chloroform-isoamyl alcohol (25:24:1). Plasmid DNA was ethanol-precipitated and dissolved in 1 μ l TE. The following oligonucleotides were used for this primer extension reaction: (1) M13 Reverse Sequencing Primer (5'-AGCGGATAACAATTTCACACAGGA-3'), which is complementary to single-stranded prepared in vivo,

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5'-caccacggcttaccacttattgctccgctcgtattgttgggaattgagcgggaacaaattccacaggaacacgtatg
                                     M13 Reverse Sequencing Primer
acgtgtatcgaattatcagctactcatatggaatttggccctctcgaggcccaagaattcccgacttaccgtat
T7 Promoter                               SfiI                               EcoRI                               SmaII
GTCCGGGATCCGCTCTTAATTAAAGCGGCCGCAAGCTTttccctattgpggggttaatttatcgtggcgc
BamHI                               PacI                               HindIII                               T3 Promoter
tggcgcctgttttaccatgctcgagctgggaacaccctgccttaccacacttaactgccttcgag3'
                                     M13 Sequencing Primer
```

Figure 6 Sequence of the pT773-*Pac* polylinker (uppercase) and flanking sequences (lowercase).

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and (2) Oligo-Amp (5'-GACTGTGAGTACTCAAC-CAAGTC-3'), which is complementary to the ampicillin resistance gene of single-stranded pT73-Pac or Lactid BA plasmids prepared in vivo.

In Vitro Synthesis of Library RNA

Some 2–5 µg of double-stranded plasmid DNA from either the starting library (see methods 2-1 and 2-2 below) or the mini-library of abundant cDNAs (see method 2-3 below) was linearized with either *PacI* (NEB) or *NotI* (NEB) and used as a template for synthesis of RNA with RiboMax Large Scale RNA Production Systems T7 or T3 (Promega), according to the manufacturer's instructions. After treatment with ribonuclease-free DNase I (Promega), to digest away the plasmid DNA template, the RNA was used for hybridization as described below. It should be noted that RNA synthesized with T7 RNA Polymerase is in the message-like orientation and is complementary to the single-stranded circles produced in vivo. On the other hand, RNA synthesized with T3 RNA Polymerase is in the antisense-like orientation and it is complementary to single-stranded circles produced in vivo.

Normalization Method 1

The procedure used for construction of the normalized human infant brain (INIB) library (here designated as method 1) has been described previously (Sorensen et al. 1994). Method 1, with minor modifications, was also applied to construct the normalized human fetal liver/spleen cDNA library (INFLS). To synthesize a partial second strand of about 200 nt by limited extension, a 100 µl reaction mixture containing 5 µl 0.5 µg/µl *PvuII*-digested, HAP, and gel-purified single-stranded plasmid DNA from the fetal liver/spleen starting library, 7 µl 10 µg/µl oligo (dT)₁₂₋₁₈ (Pharmacia), 10 µl 10× Primer Extension Buffer [0.3 M Tris (pH 7.5), 0.5 M NaCl, and 0.15 M MgCl₂], 10 µl 0.1 M DTT, 10 µl mixed dNTP stock, 25 µl mixed ddNTP stock (a solution containing each dideoxy A, C, and G at a final concentration of 25 mM), 5 µl 800 U/µl *Pol*^{II} (Pfu), and 20.5 µl water was incubated at 60°C for 5 min, at 50°C for 15 min, and at 37°C for 2 min. Then 7.5 µl 5 units/µl Klenow enzyme (USB) was added, and the reaction was incubated at 37°C for 30 min. The reaction was extracted with phenol-chloroform-isoamyl alcohol (25:24:1), 5 µg methanol and sheared salmon sperm DNA was added, and the partially double-stranded plasmids were purified from the remaining single-stranded circles (unprimed molecules, as well as clones derived from mRNAs with an internal *PacI* site that therefore do not contain an oligo(dA) tail at the 3' end) by HAP chromatography. The HAP-bound fraction containing the partially double-stranded plasmids was eluted with 6 ml 0.4 M sodium phosphate buffer (pH 6.8), 10 mM EDTA, and 1% SDS, and plasmid DNA was desalted as described before (Sorensen et al. 1994) and ethanol-precipitated. The DNA (173 ng) was resuspended in 2.5 µl deionized formamide and melted at 80°C for 3 min under 10 µl mineral oil. Then 1 µl of 5 µg/µl oligo(dT)₁₂₋₁₈ was used to block the tails) was added, and the mixture was heated at 80°C for 1 min. Then 0.5 µl 5 M NaCl, 0.5 µl 10× TE, and 0.5 µl water were added, and the reassociation reaction was incubated at 42°C for 0.6 hr

(calculated $C_0t = 0.5$). The remaining single-stranded circles were purified over HAP (flow-through fraction) and subjected subsequently to a second cycle of the normalization procedure as described above, except that reassociation was conducted for 24 hr (calculated $C_0t = 20$). The remaining single-stranded circles (normalized library; INFLS) were purified over HAP, converted to double-stranded plasmids, electroporated into DH10B bacteria, and propagated under ampicillin selection.

Normalization Methods 2-1, 2-2, and 2-3

Method 2 is a reassociation kinetics-based approach involving hybridization of in vitro synthesized RNA (the driver) derived either from the entire library (methods 2-1 and 2-2; see Fig. 2) or from a mini-library enriched for abundant cDNAs (method 2-3; see Fig. 2), with the whole starting library in the form of single-stranded circles (the tracer). The remaining single-stranded circles (normalized library) are purified by HAP chromatography (HAP flow-through fraction), converted to double-stranded plasmids for improvement of electroporation efficiency, electroporated into DH10B bacteria (Life Technologies), and propagated under ampicillin selection. A number of normalized cDNA libraries were constructed with these methods using single-stranded plasmids prepared both in vivo and in vitro (see Table 1). In all three variants, the driver was first pre-annealed with a pair of oligonucleotides to block both 5' and 3' vector sequences as follows: 0.5 µl (10 µg) of each oligonucleotide, 1 µl RNA (5.0 µg in methods 2-1 and 2-2; 0.5 µg in method 2-2), and 4.0 µl deionized formamide were heated for 3 min at 80°C under 10 µl mineral oil and quickly chilled on ice. Then 0.8 µl 10× hybridization buffer [0.4 M Pipes (pH 6.4), 4 M NaCl, and 10 mM EDTA in methods 2-1 and 2-2; 0.4 M Pipes (pH 6.4), 1.2 M NaCl, 10 mM EDTA, and 1% SDS in method 2-2), 0.5 µl RNasin (Boehringer Mannheim), and 0.7 µl water were added and the mixture (total volume, 8 µl) was incubated overnight at 42°C (methods 2-1 and 2-2) or 30°C (method 2-2). In another tube, 2.5 µl (50 ng) single-stranded library DNA in deionized formamide was heated for 3 min at 80°C under mineral oil; 0.5 µl 10× hybridization buffer and 2.0 µl water were added, and the mixture was transferred to the tube containing the preannealed RNA. Hybridization (13-µl reaction) was performed at 42°C (method 2-1; $C_0t = 5-10$; method 2-2; $C_0t = 100-200$) or at 30°C (method 2-2; $C_0t = 5-10$). The driver, rather than the tracer, was blocked because otherwise the latter would, to some extent, bind to HAP during purification. The plasmid mini-library enriched for abundant cDNAs that served as a template for the synthesis of RNA used as driver in method 2-3 was prepared from the HAP-bound fraction obtained during purification of the normalized library in method 2-1. Different pairs of blocking oligonucleotides were used, depending on whether the RNA was synthesized with T3 or T7 RNA polymerases. To block RNA synthesized with T3 RNA polymerase, which was used in hybridizations with single-stranded plasmids prepared in vivo we used: 5'-³²P-AUGCGCGCCGCAAGCTATTCCCTTTAGT-GAGGTTAAAT-3' (this oligonucleotide was used to block 5' vector sequences of all but the human fetal liver/spleen library RNA), and 5'-³²P-AGATTTTATTAATGAAGCGCGC-CAGCTATTCCCTTTAGTGAAGGTTAAAT-3' (this oligonucleotide was used to block 5' vector sequences of the

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human fetal liver/spleen library RNA), and 5'-AGG-CGAAGATTGGGACAGG-3' (this oligonucleotide was used to block 3' vector sequences). To block RNA synthesized with T7 RNA polymerase, which was used in hybridizations with single-stranded plasmids prepared in vitro we used: 5'-CCTCTGGTGGGCAATTCCTGGCCCTCAG-GGGCAATTCGC-3' (this oligonucleotide was used to block 5' vector sequences). The oligonucleotide used to prime the synthesis of first-strand cDNA was also used to block 3' vector sequences.

Normalization Method 3

Method 3, used to generate the normalized library from multiple sclerosis plaques (DNILMS), is a reassociation-kinetics based approach involving hybridization (C_{0t} = 20–25) of a 20-fold excess of Exonuclease III-digested cDNA inserts excised from a plasmid DNA preparation of the starting library with the library itself in the form of single-stranded circles, followed by HAP-purification of the remaining single-stranded plasmids, conversion to double-strands, and electroporation into bacteria. Some 5 µg double-stranded plasmid DNA from the starting library was doubly digested with *NciI* and *NotI*; the excised cDNA inserts were separated from the cloning vector by agarose gel electrophoresis; and the DNA was purified using beta-agarose (NEB) according to the manufacturer's instructions. Then 0.6 µg gel-purified double-stranded cDNA inserts in 47.5 µl TE was digested with Exonuclease III at 37°C for 30 min in a 60-µl reaction containing 6 µl 10× Exonuclease III buffer (0.5 M Tris (pH 8.0) and 50 mM MgCl₂), 0.6 µl 0.1 M DTT, 2.9 µl water, and 3 µl of 65 units/µl Exonuclease III (Life Technologies). The Exonuclease was then digested with 136 µg Proteinase K (Boehringer Mannheim) at 50°C for 15 min in a 100-µl reaction containing 10 mM Tris (pH 7.8), 5 mM EDTA, and 0.5% SDS. After two extractions with phenol-chloroform-isoamyl alcohol (25:24:1), the resulting noncomplementary single-stranded DNA (total amount ~0.3 µg) was ethanol-precipitated and resuspended in 1 µl TE. A 5-µl hybridization reaction was then set up as follows: 1 µl Exonuclease III-digested cDNA inserts (an estimated amount of 150 ng of single-stranded DNA) and 20 ng single-stranded cDNA from the starting multiple sclerosis plaques library (prepared in vitro) in 2.5 µl deionized formamide were mixed and heated at 80°C for 3 min under 10 µl mineral oil. Then 0.5 µl (10 µg) of a blocking oligonucleotide (5'-CCTCTGGTGGGCAATTCCTGGCCCTCAGG-CGAAGATTGGGACAGGATTCGATTA-3'), 0.5 µl 5 M NaCl, and 0.5 µl 10× TE were added, and the mixture was incubated at 42°C for 41 hr (calculated C_{0t} of 23). The remaining single-stranded plasmids were purified by HAP chromatography, converted to double-stranded plasmids, and electroporated into DH10B bacteria (Life Technologies) as described above.

Normalization Method 4

This is a reassociation-kinetics-based approach involving hybridization of a 20-fold excess of cDNA inserts generated by PCR with the library itself in the form of single-stranded circles, followed by HAP purification of the remaining single-stranded plasmids, conversion to double-strands,

electroporation into DH10B bacteria, and amplification under ampicillin selection. PCR amplification of cDNA inserts was performed using the Expand High Fidelity PCR System (Boehringer Mannheim) according to the manufacturer's instructions. This PCR system is composed of an enzyme mixture containing thermostable Taq DNA and Pwo DNA polymerases (Barnes 1994). An amount of 1 µl (2.5–5.0 ng) DNA template [double-stranded plasmids (fetal lung, parathyroid adenoma, senescent fibroblasts) or single-stranded circles prepared in vitro (fetal heart, 14Nb2HLS20W-fetal liver/spleen, and all mouse, rat, and schistosome libraries listed in Table 1)] was mixed with 2 µl dNTP stock (the final concentration of each dNTP in the reaction is 200 µM), 5 µl of a 20-µM solution of T7 Primer (5'-TAATACGACTCAGTATAGG-3'), 5 µl of a 20-µM solution of T3 Primer (5'-ATTACCGCTCAGTAAGGA-3'), 10 µl 10× Expand High Fidelity buffer, 0.75 µl Expand High Fidelity enzyme mix (2.6 units), and 76.25 µl water. Then 50 µl mineral oil was added and the reaction mixture was subjected to the following amplification cycle conditions in a Perkin Elmer Thermocycler: 7 min while ramping up from room temperature to 94°C, 20 cycles of 1 min at 94°C, 2 min at 55°C, and 3 min at 72°C, and 7 min at 72°C. PCR-amplified fragments were purified using the High Pure PCR Product Purification Kit (Boehringer Mannheim) as instructed by the manufacturer. The purified PCR product was ethanol-precipitated and dissolved in 5 µl TE. Then 1.5 µl (0.5 µg) PCR products was mixed with 5 µl (50 ng) library DNA (single-stranded circles prepared in vitro) in deionized formamide, 0.5 µl (10 µg) 5' blocking oligo AV-1 (5'-CCTCTGGTGGGCAATTCCTGGCCCTCAGGCGCAAAATTCCTATAGTGAGTCGTATTA-3'), 0.5 µl (10 µg) 3' blocking oligo AR (5'-ATTACCGCTCAGTAAGGAATAGCTGTCGGCGCGCTA-3'), used for all but the fetal liver/spleen library, or alternatively, (0.5 µl (10 µg) 3' blocking oligo AV-2 (5'-ATTACCGCTCAGTAAGGAATAGCTGTCGGCGCGCTAATATTAAGATCTT-3'), used only for the fetal liver/spleen library), and this mixture was heated at 80°C for 3 min under 10 µl of mineral oil. Then 1 µl 10× buffer-A [1.2 M NaCl, 0.1 M Tris (pH 8.0), and 50 mM EDTA; used for fetal lung, fetal heart, parathyroid adenoma, senescent fibroblasts, and 19.5-days postconception (dpc) mouse embryo] or, alternatively, 1 µl 10× buffer-B [1.2 M NaCl, 0.1 M Tris (pH 8.0), and 10% SDS; used for 14Nb2HLS20W-fetal liver/spleen, 17.5-dpc mouse embryo, 13.5- to 14.5-dpc mouse embryo, rat heart, rat kidney, and 8-week schistosome], and 1.5 µl water were added, and the hybridization was performed at 30°C for 24 hr (calculated C_{0t} = 5). The remaining single-stranded circles were purified by HAP chromatography, converted to double-strands, and electroporated into DH10B (Life Technologies) bacteria, as described above.

Subtractive Hybridization

Double-stranded plasmid DNA from a pool of 4992 clones grown individually in 384 well plates (IMAGE Consortium plates LLAM 78-90, identification nos. 66696–67079 and 108168–112775) derived from the normalized fetal liver/spleen library (DNLS) was prepared using the Qiagen Mid-prep kit according to the manufacturer's instructions, and converted to single-stranded circles in vitro, as described above. Single-stranded circles were purified by

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HAP chromatography and used as a template for PCR amplification with T7 and T3 primers, as described above. An amount of 1.5 µg of PCR-amplified cDNA inserts from the LLAM 78-90 pool (in 4 µl deionized formamide) was mixed with 50 ng of single-stranded circles from the INFLS library (in 2 µl deionized formamide), 2.1 µl (42 µg) 5' blocking oligo AV-1, and 2.1 µl (42 µg) 3' blocking oligo AV-2. Then 10 µl mineral oil was added, and the mixture was heated at 80°C for 3 min. Then 1.2 µl 10× buffer-B and 0.6 µl water were added, and the hybridization was performed at 30°C for 48 hr (calculated $C_{it} = 27$). The remaining single-stranded circles were purified over HAP, converted to double-strands, electroporated into DH10B bacteria, and propagated under ampicillin selection to generate the subtracted liver/spleen library (INFLS-S1). HAP-bound DNA was also processed and purified for use in control experiments.

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